THE EFFECT OF DIFFERENT GIEMSA STAINING CONDITIONS ON THIN BLOOD FILM MALARIA IDENTIFICATION

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Abstract. Malaria is a major public health problem in many countries, including Thailand. The gold standard of malaria diagnosis and species identification is microscopic examination but this depends on the skill of the microscopist and the quality of the Giemsa stained blood smear. Different concentrations of Giemsa and staining times may affect dye deposits interfering with malaria identification and resulting in false positive findings since the dye deposit size is similar to that of malaria. The purpose of this study was to determine the ideal Giemsa stain concentration and staining time to identify malaria on a thin blood film with the lowest dye deposits. Positive and negative blood films were prepared and stained with Giemsa with following regimens: 2.5% for 45 min, 2.5% for 60 min; 3% for 30 min, 3% for 40 min; 5% for 20 min, 5% for 30 min; 10% for 10 min, 10% for 20 min, 10% for 30 min; 20% for 5 min, 20% for 10 min, 20% for 20 min, 20% for 30 min; 30% for 5 min and 30% for 10 min. Each slide was reviewed by 3 microscopists in triplicate who were blinded to whether the slide was positive or negative. One slide was made for each regimen giving a total of 270 observations of that 30 slides (15 for negative and 15 for positive) by the 3 microscopists in triplicate. Of these, 4.8% had false positive results and 0% had false negative results. False positive results were: 1 positive result each at 10% for 20 min, 10% for 30 min, 20% for 5 min, and 2 positive results each for 20% for 10 min, 20% for 20 min, 20% for 30 min, 30% for 5 min and 30% for 10 min. Our results show the greater the concentration, the greater the false positive rate. Therefore, the most efficient staining regimen in our study was 3% for 30 min which give a 100% sensitivity and 100% specificity. The concentration and time had the fewest dye deposits and was still able to detect the malaria species.

Keywords: malaria, stained blood smear, Giemsa, concentration

INTRODUCTION

Malaria is caused by *Plasmodium* spp, comprised of 6 species that infect humans: *Plasmodium falciparum*, *P. vivax*, *P. ovale curtisi*, *P. ovale walliken*, *P. malariae* and *P. knowlesi* (Cowman *et al*, 2016). In 2016, there were an estimated 216 million malaria cases worldwide with 445,000 deaths (WHO, 2016). The Bureau for Vector-borne Diseases, Department of Disease Control, Ministry of Public Health, Thailand reported 3,483 malaria cases in Thailand from 1 January to 19 September 2016

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(Bureau of Vector-borne Discases, 2016).

Correct malaria species identification is important to inform treatment (WHO, 2012). Two methods are commonly used to diagnose malaria infection. The first is microscopic examination, which is the standard reference method for malaria diagnosis and the second is a rapid diagnostic test. Microscopic examination is inexpensive and gives an estimate of parasite numbers and identifies the species and stage of development of the parasite, which allows monitoring of parasite clearance during treatment. Stained blood smears can also serve as a permanent record for future reference. A disadvantage of microscopy is that it requires a microscopist with a high level of expertise. A blood film can identify ≥ 4 parasites/µl blood depending on the quality of the stained blood smear (Murphy *et al*, 2013). Guidelines for the Laboratory Diagnosis of Malaria state Giemsa or Leishman stain should be used for thin films and Giemsa or Field stain should be used for thick films (Bailey *et al*, 2013). One study compared Leishman with Giemsa stains for thick and thin blood films for diagnosing malaria and found that Leishman stain is a good alternative for identify malaria and gives good visualization of red blood cell and white blood cell morphology (Sathpathi *et al*, 2014) but Giemsa stain is more commonly used and gives good visualization of the malaria parasite morphology and organelle details allowing better identification of the species of the malaria (CDC, 2016).

In Thailand, Giemsa is the most commonly used stain to identify malaria on thick and thin blood films. The recommended regimens for preparing thick and thin blood smears for malaria diagnosis are 2.5% Giemsa stained for 45-60 min (CDC, 2016), 4-5% concentration of Giemsa stained for 20 min for thick blood smears and 5-10% Giemsa stained for 20 min for thin blood smear (Malaria Research and Reference Reagent Resource Center, 2013). Other recommended regimens include 10% Giemsa stained for 8-10 min for a blood smear prepared within 1 day of collection, 3% Giemsa stained for 30 min for a blood smear prepared greater than 1 day of collection (Bureau of Vector-borne Disease, 2009) and 20% Giemsa for 30 min for thin blood films (Chanhoklong *et al*, 2014). Different concentrations of Giemsa stain and varying staining times can affect the quality of the stained blood smear. High concentrations of Giemsa stain increase the contrast but may also increase unwanted dye deposits which can interfere with malaria identification and give false positive results since the dye deposits are similar in size to malaria parasites. Therefore the objective of this study was to investigate various Giemsa stain concentrations and staining times to determine the best staining regimen to identify malaria.

MATERIALS AND METHODS

Blood smear preparation

Thin blood films from blood with (positive) and without (negative) malaria were used for this study. For the positive blood smear, 2.5% *P. falciparum* parasitemia from malaria culture was used. Each slide was air-dried first and then fixed with methanol for 30 seconds.

Giemsa stain preparation

Stock Giemsa stain was prepared with 270 ml absolute methanol (RCI Labscan, Bangkok, Thailand), 3 g Giemsa stain powder (LOBAChemie, Mumbai, India) and 140 ml glycerol (Ajax Finechem, Bayroad, Taren Point, NSW, Australia) in a brown bottle with glass beads and a screw cap. The ingredients were shaken for 30 - 60 min daily for 14 days before use. The Giemsa

Table 1
Concentrations of Giemsa stain and staining times.

Concentrations of Giemsa stain (%)	Staining times (min)
2.5	45, 60
3	30, 40
5	20, 30
10	10, 20, 30
20	5, 10, 20, 30
30	5, 10

stain was then filtered through a Whatman No.1 filter (GE Healthcare, Wuxi, China) before use (CDC, 2016).

Blood smear staining

Giemsa stain with 2.5%, 3%, 5%, 10%, 20%, and 30% concentration were prepared. The lengths of time used to stain the slide at each concentration are shown in Table 1. Only thin smears were used in this study, not thick smears.

Examiners

Three microscopists trained in identifying malaria by microscopy were used for the exam. Each microscopist examined each slide in triplicate and was blinded to whether the slide was negative or positive for malaria. Each microscopist also graded the quantity of unwanted dye deposits from 1 (no unwanted dye deposits) to 5 (a large number of unwanted dye deposits).

Statistical analysis

Differences of quantities of unwanted dye and parasitemia on the thin smears were analyzed using the Kruskal-Wallis test and Mann-Whitney *U* test. The correlation between the percent parasitemia and level of dye deposits was analyzed with the Spearman's rho. A p-value < 0.05 was considered statistically significant. The Statistical Package for the Social Sciences (SPSS), version 17.0 (IBM, Armonk, NY) was used to make the calculations.

Ethical considerations

This study was approved by the Human Ethics Committee of Naresuan University (No. NU-IRB 453159).

RESULTS

Quantification of unwanted dye deposits

Positive and negative thin blood films were prepared and stained with Giemsa with the following regimens: 2.5% for 45 min, 2.5% for 60 min; 3% for 30 min, 3% for 40 min; 5% for 20 min, 5% for 30 min; 10% for 10 min, 10% for 20 min, 10% for 30 min; 20% for 5 min, 20% for 10 min, 20% for 20 min, 20% for 30 min; 30% for 5 min and 30% for 10 min. The different levels of unwanted dye deposits by stain concentrations and staining times are shown in Fig 1. The largest number of unwanted dye deposits occurred with 30% Giemsa stained for 10 min and the lowest number occurred with 3% Giemsa stained for 30 min. The level of unwanted dye deposits in all Giemsa staining condition were significantly different ($p<0.05$) by Kruskal-Wallis statistical analysis. The level of unwanted dye deposits in each Giemsa condition was compared with 3% Giemsa for 30 min, the lowest number of unwanted dye deposites. The results of analysis by Mann-Whitney *U* test showed that there were significant differences between 3% Giemsa for 30 min and 2.5% for 60 min, 5% for 20 min, 5% for 30 min, 10% for 10 min, 10% for 20 min, 10% for 30 min, 20% for 5 min, 20% for 10 min, 20% for 20 min, 20% for 30 min, 30% for 5 min and 30% for 10 min ($p<0.05$) as shown in Fig 1.

Malaria identification

The results of Giemsa staining showed clear malaria morphology (Fig 2-thin arrow) with chromatin stained in blue and

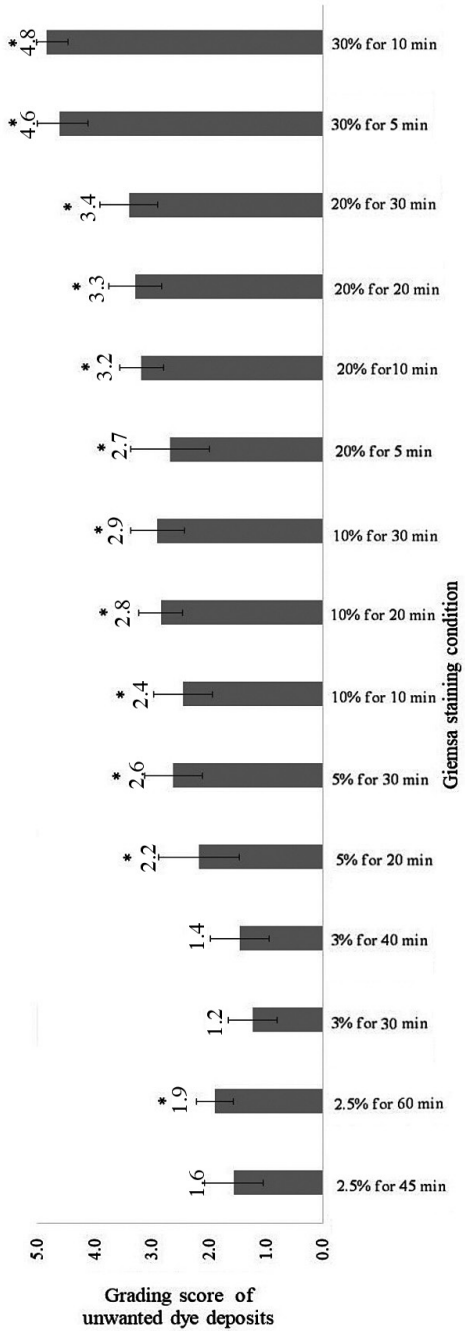

Fig 1–The grading score of unwanted dye deposits in various Giemsa stain concentrations and stain times. * $p < 0.05$.

cytoplasm stained in pink with the following regimens: 2.5% for 45 min, 2.5% for 60 min, 3% for 30 min, 3% for 40 min, 5% for 20 min, 5% for 30 min, 10% for 10 min, 10% for 20 min, 10% for 30 min, 20% for 20 min, 20% for 30 min and 30% for 10 min. A large number of dye deposits (Fig 2- thick arrow) were seen with 30% Giemsa stained for 10 min. There were 13 false positives (4.8%) (Table 2) found with 10% Giemsa stained for 20 min, 10% for 30 min, 20% for 5 min 20% for 10 min, 20% for 20 min, 20% for 30 min, 30% for 5 min and 30% for 10 min. One hundred percent sensitivity and specificity were seen with 2.5% Giemsa stained for 45 min, 2.5% for 60 min, 3% for 30 min, 3% for 40 min, 5% for 20 min, 5% for 30 min and 10% for 10 min. A sensitivity of 88.9% was seen with 20% Giemsa stained for 10 min, 20% for 20 min, 20% for 30 min, 30% for 5 min, and 30% for 10 min (Table 3). This was due to the large number of unwanted dye deposits.

Parasitemia determination

The percentage of parasitemia by staining regimens are shown in Fig 3. There was high percentage of parasitemia in the condition with high concentration of Giemsa staining. The highest value of parasitemia was from the condition in which 30% Giemsa staining for 10 min (3%). There were significant differences of parasitemia in thin blood smears staining with different Giemsa concentrations and staining times. The percent parasitemia in each Giemsa condition was compared with 3% Giemsa for 30 min, the condition giving 100% sensitivity and specificity. The result showed that there were significant difference of percent parasitemia by Mann-Whitney *U* test, between 3% Giemsa for 30 min and 20% for 30 min, 30% for 5 min and 30% for 10 min as shown in Fig 3. In addition, there was correlation between percentage of parasitemia and number of dye deposits by Spearman's rho at $p < 0.05$ (r=0.379).

DISCUSSION

We studied the best Giemsa staining regimen to detect malaria on thin films, and

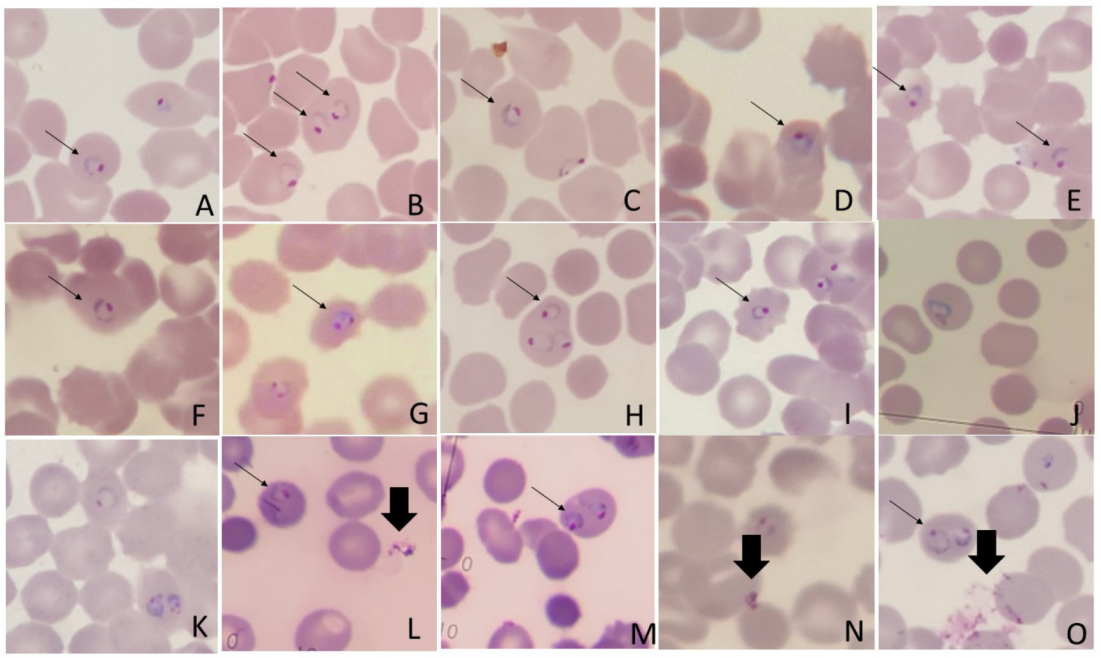

Fig 2–Malaria parasites (thin arrow) and unwanted dye deposits (thick arrow) seen with the following Giemsa staining regimens: A) 2.5% for 45 min, B) 2.5% for 60 min, C) 3% for 30 min, D) 3% for 40 min, E) 5% for 20 min, F) 5% for 30 min, G) 10% for 10 min , H) 10% for 20 min, I) 10% for 30 min, J) 20% for 5 min, K) 20% for 10 min, L) 20% for 20 min, M) 20% for 30 min, N) 30% for 5 min, O) 30% for 10 min.

Table 2
Percentage of false positive malaria slides for the 270 observations.

Giemsa staining conditions	Percent false positive for malaria
2.5% for 45 min	0
2.5% for 60 min	0
3% for 30 min	0
3% for 40 min	0
5% for 20 min	0
5% for 30 min	0
10% for 10 min	0
10% for 20 min	0.37
10% for 30 min	0.37
20% for 5 min	0.37
20% for 10 min	0.74
20% for 20 min	0.74
20% for 30 min	0.74
30% for 5 min	0.74
30% for 10 min	0.74
Total	4.8

found it to be 3% Giemsa stained for 30 min which give a 100% sensitivity and specificity and had the lowest number of unwanted dye deposits, which can be mistaken for malaria. To our knowledge, there are no any other published studies of different Giemsa staining regimens for detecting malaria on thin blood film, the effect of unwanted dye deposits on malaria identification and percent parasitemia in thin blood film. One study evaluated the quality of staining by different regimens but did not compare the parasitemia determination (Chanhoklong *et al*, 2014). Unwanted dye deposits can interfere with malaria identification, since they may look similar to malaria parasites resulting in incorrect of identification of parasitemia. Misdiagnosis is more likely happen, at lower parasite densities, as noted in a

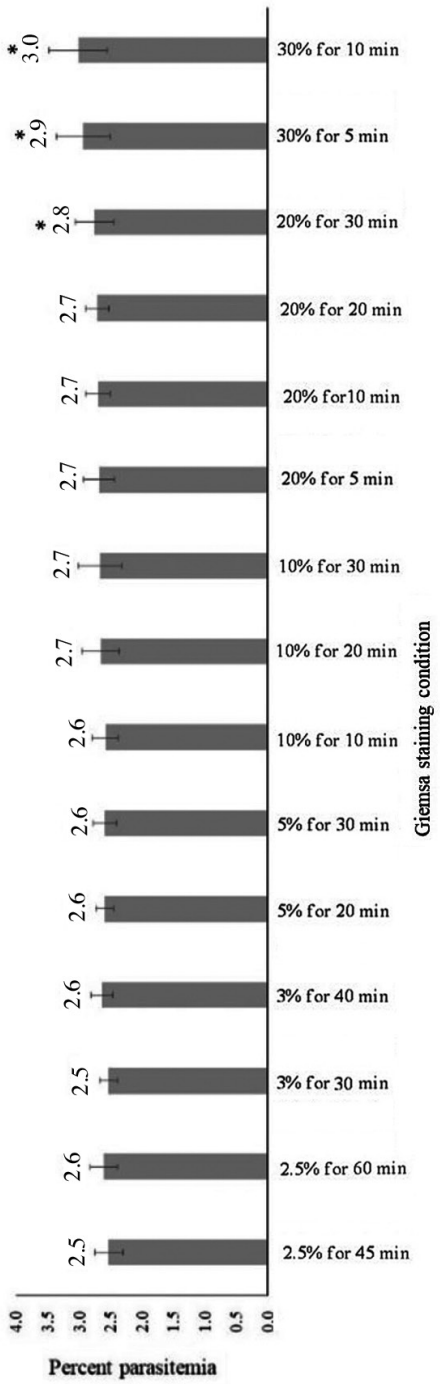

Fig 3–The percent parasitemia in thin blood smear staining in different concentrations of Giemsa stain and staining times.
*$p < 0.05$.

previous study (Maguire *et al*, 2006). Stain quality can also result in the misidentification of malaria parasite species, especially in distinguishing *P. vivax* from *P. ovale* due to their similar morphology (Bailey *et al*, 2013). In our study, we only used *P. falciparum*. This is due to the difficulty in culturing other malaria species in the laboratory. *P. falciparum* is the most virulence species found in Thailand (Bureau of Vector Borne Diseases, 2016). Therefore, we studied *P. falciparum*. Further studies need to be investigated by using blood from malaria infected patients.

In our study, we only used experienced, trained microscopists. A previous study compared malaria identification between experienced and inexperienced microscopists and found agreement of participants in detection of malaria parasites was better than the agreement in the identification of different species of malaria. Poor agreement was reported in the detection of parasites at low density or with mixed infections (Ayalew *et al*, 2014).

Performing both thick and thin blood smears is the gold standard for malaria diagnosis (Bailey *et al*, 2013) and it is recommended to examine at least 200 high power fields on thick smear to rule out malaria (Bailey *et al*, 2013). When a thick blood smear is positive for malaria then a thin blood smear should be performed to identify the malaria species (Bailey *et al*, 2013). In our study, only thin blood films were performed. Therefore, the effect of different concentrations of Giemsa and staining times in thick blood films need to be investigated.

In summary, we recommend using 3% Giemsa stain for 30 min for thin film smears which give 100% sensitivity and specificity for detecting malaria with fewest number of unwanted dye deposits, which can result in false positives.

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Table 3
Sensitivities and specificities for detecting malaria with various Giemsa staining regimens.

Giemsa staining conditions	Sensitivity	Specificity
2.5% 45 min	100	100
2.5% 60 min	100	100
3% 30 min	100	100
3% 40 min	100	100
5% 20 min	100	100
5% 30 min	100	100
10% 10 min	100	100
10% 20 min	100	94.4
10% 30 min	100	94.4
20% 5 min	100	94.4
20% 10 min	100	88.9
20% 20 min	100	88.9
20% 30 min	100	88.9
30% 5 min	100	88.9
30% 10 min	100	88.9

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