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Comparing Of Staining Giemsa Dilutions For Rapid Detection Of Malaria Parasites At Thick And Thin Blood Smears In Biak And Abepura General Hospitals, Papua, Indonesia

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Abstract

Background. The Giemsa stain has been used as gold standard of staining blood smears for diagnosis malaria parasites. Method of Microscopic examination of thick and thin blood smears could be developed become an accurate methods with the effective time so that the patients can be take result examination.

Objective. To determination and evaluation a rapid methods of the diagnosis of malaria infection by microscopic of stained thin and thick blood smears with modification staining of Giemsa dilutions were compared with standard Giemsa based on time.

Methods. This research are quasi experiment study with observation design just looking for three methods are effective with five different times which used in microscopy examination of malaria parasites. The Sample thin and thick blood smears were stained with modification staining of 1:4, 1:5 and 1:6 Giemsa dilution and compared with standard Giemsa staining 1:3, 3% and 10%, with ph 7.2 based on time.

Result. A total of 130 malaria positive cases, 65 (50) patients were found to carry P. falciparum and 65 (50) carried were P.vivax. The results of this study was indicated that the higher the dilution of the Giemsa solutions, the reduced parasite of malaria.

Conclusion. Giemsa dilution method that can be used for staining malaria preparations, thick and thin blood smears rapid and accurately in detecting the parasites density of malaria was 1:4 with a duration time of 15 - 20 min, while the 1:5 and 1:6

methods were staining time 5 - 10 min cannot be used because the parasites density of malaria was reduced as result of incomplete lysed of erythrocytes and half parasites were covered by Giemsa.

Keywords: Giemsa, Blood smears, P. falciparum, P. vivax

Introduction

Malaria is a major health problem in the world, and causes 229 million infections and 409.000 deaths worldwide [1,2] and especially in Indonesia, the morbidity tare reflected in Annual Parasites Incidence was found 0.93 per 1000 in 2019, this number has increased from 2018 which was 0.84 per 1000 population. The highest malaria morbidity rate in Papua is 64.03per 1000 population in 2019 [3]. Rapid and accurate diagnosis of malaria parasites is an essential strategy for effective cases of malaria management as well as the public health response to malaria.

There are four major Plasmodium species infecting humans in Papua; P. falciparum, P.vivax, P.ovale and P.malariae [4] but P. falciparum and P. vivax are two main causes of human malaria infections. Falciparum malaria poses can be caused of severe complicated and majority patients of deaths [5]

The Giemsa stain was still used as the gold standard to detect malaria parasites in thick and thin blood smears in the world [6]. In clinical laboratory, a properly stained blood films were critical for malaria diagnosis, especially for identify of Plasmodium species.

Giemsa stain have been recommended and most reliable procedure for staining thick and thin blood smears. Composition of Giemsa is eosin and methylene blue (azure). The eosin for stains the parasite nucleus red, while the methylene blue for stain the blue cytoplasm. The thin blood smear is fixed with methanol. Malaria could be causes morbidity and mortality in the world [7.8]

Malaria is diagnosed microscopically by staining thick and thin peripheral blood smears on an object glass. Microscopy of standard Giemsa stained thick and thin blood smears still the current gold standard for clinical laboratory diagnosis in clinical and education laboratories.[2] The standard staining time for Giemsa 3% there is between 45 - 60 min that will be compared with modification staining methods time we used 5 - 10 min, 10 -15 min, 15 - 20 min, 20 -25 and 25 - 30 min for identified species of malaria parasites and from five times we will looking for a rapid Giemsa stained time of three Giemsa staining dilutions [6,7]

Parasite density serves as one of the diagnostic criteria for severe malaria infection and monitoring is important for the diagnosis and treatment of malaria patients. Parasite density was associated with prognosis of patients [2,9]

The aim of this research was found to compare the Giemsa staining dilution which quality in thick and thin blood smears of malaria parasites when stained for different times and concentrations to optimize the Giemsa for rapid staining. The three different modified staining of Giemsa dilutions were used to stain thick and thin blood smears of patients are infected by P. falciparum and P. vivax with comparing stain of 1:4, 1:5, 1:6 dilutions for detection morphological difference between species of malaria parasites in infected erythrocytes with five different times and we are looking for concentration staining that accurate with a time which rapid so that the patients can be helped with soon.

In this research, we were making for describe a modification of the traditional Giemsa staining method and compared both methods for identification morphological of malarial parasites species and erythrocytes based on rapid diagnosis time [10]

Methods and Study Site

A total of 130 patients from Biak and Abepura General Hospitals were infected by Plasmodium falciparum and P.vivax. These patients presented at the clinical laboratory of Biak and Abepura general hospitals with fever. The diagnosis of malaria infection was confirmed by presence of malarial parasites in thick and thin blood smears stained with modification staining of Giemsa dilutions and compared with standard Giemsa staining methods.

Giemsa staining. A 3 % Giemsa stain was prepared by taking 3 mL of Giemsa stain (Fluka chemicals, No.48900, Switzerland) in 97 mL of phosphate buffer (pH7.2) and filtering the stain. A fresh stain was made every 3rd day. The blood smears were stained with 3% Giemsa stain for 45-60 min as standard Giemsa staining [11,12]

Giemsa staining. A 10 % Giemsa stain was prepared by taking 10 mL of giemsa stain (Fluka chemicals, No.48900, Switzerland) in 90 mL of phosphate buffer (pH7.2) and filtering the stain. A fresh stain was made every 3rd day. The blood smears were stained with 10% giemsa stain for 10-15 min as standard Giemsa staining [12,13]

Giemsa staining. A 1:3 Giemsa stain was prepared by taking 3 drops of giemsa stain (Fluka chemicals, No.48900, Switzerland) in 1 mL of phosphate buffer (pH7.2) and filtering the stain. A fresh stain was made every 3rd day. The blood smears were stained with 1:3 giemsa dilutions for 30-45 min as standard Giemsa staining. [12,13,14]

Modified Giemsa staining. A Thick blood smears were made on one third of the slide, and a thin blood smears were spread on the rest of the same slide. The blood smears were air-dried for 2 - 3 min.[6] The thick blood smears were fixed and direct examined but the thin blood smears were fixed in

methanol 20 - 30 second. Immediately after fixation the slides, were placed in the filtered Giemsa stain for 5 -10 min,10 - 15 min, 15 - 20 min, 20 - 25 min and 25 - 30 min, respectively. After all, the slides were washed by three gentle dips in phosphate buffer (pH 7.2) and placed upright to air dry.

Stained thin blood smears. Preparation of the smear and the staining is similar to that used for hematology examination and Giemsa stain is used and dilution is made in alkaline buffer (pH 7.2) and now the Giemsa stain is used by most for malariology laboratories in the world [6,14]

Stained thick blood smears. Thick blood smear allow a rapid examination with large volume of blood, for detection of even parasite density in patient blood. A well prepared thick blood smear will be given more than a 10 fold increase in sensitivity more than thin smear [12].The parasites of malaria in the smear was stained with little interference in the large numbers of red blood cells present, can be seen against a relatively clear background.

Examination of slides. This research was conducted using Giemsa 1:4, 1:5 and 1:6 dilutions methods, respectively. And the results of the examination were compared with 3% Giemsa staining with a long staining time of 10 - 15 min, 15 - 20 min and 20 - 25 min, respectively. The blood smears were also examined for staining characteristic of the smear as a whole and of malarial parasites of different stages and species and the turnaround time that we were reporting in the results. In screening for malaria parasites, we used microscopic for evaluate each blood smear for its dilution staining pattern, speed and case of reading the blood smears. The parasites density in the thick blood smears were calculate by counting the number of parasites per 200 white blood cells [10, 14]

Method for staining individual slides [12]

1. Lace the slides individually on the staining rack, making sure that they are not touching each other
2. Pour the stain gently onto the slides until they are totally covered. Each slide will require approximately 3 ml of stain. Avoid pouring the stain directly onto thick blood smears
3. Leave the stain on the slide for 45 - 60 min with 3% Giemsa solution and 10 - 15 min with 10% Giemsa solution and 30- 45 min with 1:3 Giemsa solution.
4. Flood the slides gently with buffered water to float off the iridescent scum on the surface of the stain. Water buffered to 7.2 pH should be poured onto the slides from then thin smear and to avoid undue disturbance and washing off of the thick smears.

5. Remove the slides one by one and place them, thick smear downwards, in a drying rack to drain and dry, making sure that the thick smear does not touch the edge of the rack

Parasite detection. We are using thick and thin blood smears taken during malariometric survey was stained with Giemsa and subsequently examined through light binocular microscopy. Parasite density was determined by accounting the number of parasites per leucocytes in 100-high-film, assuming an average of 200 leucocytes per microscopic field and 8000 leucocytes/ µl of blood. Slides were declared negative if parasite could not be detected in 100 microscopic field. The parasite count was classifield as: (+) if 1-10 parasites were found per 100 thick film fields, (++) if 11-100 parasites per 100 microscopic thick film fields, (+++) if 1- 10 parasite per one thick film fields and (++++) if more than 10 parasites per one thick film fields [15,16].

Result

Table 1. Data Comparison on malaria patients with used modified staining of Giemsa dilutions and standard Giemsa based on time in Biak and Abepura general hospitals

Time (min)	Malaria patients	Modified Giemsa Staining Dilutions						Standard Giemsa staining		Prequency (%)	Pvalue
		1:4		1:5		1:6		3%			
		Pf	Pv	Pf	Pv	Pf	Pv	Pf	Pv		
25-30	Parasites density										
	+3							3	3	6	
	+4							2	2	4	
20-25	Parasites density										
	+3	3	3	3	3	3	3			18	
	+4	2	2	2	2	2	2			12	0.000
15-20	Parasites density										

	+3	3	3	3	3	3	3			18	
	+4	2	2	2	2	2	2			12	
10-15	Parasites density										
	+3	3	3	3	3	3	3			18	
	+4	2	2	2	2	2	2			12	
5-10	Parasites density										
	+3	3	3	3	3	3	3			18	
	+4	2	2	2	2	2	2			12	
Total		20	20	20	20	20	20	5	5	130	

The result of this study showed that the modified staining of 1:4, 1:5, and 1:6 dilutions of Giemsa with between time 5-10 min,10 -15 min, 15 - 20 min, and 20 - 25 min, respectively. A ratio of 1:4, with a time of 15-20min the staining is better than the modified solution of 1:5 and 1:6 with a time of 5- 10 min. The statistic one sample t test shown, (P.value : 0.000 < 0.05, CI= 95%; 0.05). There is significant difference between the malaria parasites density and concentration of modification staining 1:4,1:5 and 1:6 dilutions of Giemsa, respectively.

Table 2. The observation data of modified staining of Giemsa dilutions for rapid diagnosis to detection malaria parasites by microscopic based on times in Biak and Abepura general hospitals

Turnaround time	Modified Giemsa staining			Parasite staining	Red blood cell Staining	
	1:4	1:5	1:6	Thick/Thin	Thick	Thin
5-10 min	10	10	10	Parasite pigment distinct	Incomplete and complete hemolysis	heterogeneous and homogenous staining
10-15min	10	10	10	parasite pigment	incomplete hemolysis	Heterogeneous Staining

				distinct		
15-20 min	10	10	10	parasite pigment distinct	Complete and incomplete hemolysis	Homogenous and heterogenous staining
20-25 min	10	10	10	parasite pigment distinct	incomplete hemolysis	Heterogeneous staining
25-30 min	0	0	0	Not founded parasite	incomplete hemolysis	Heterogeneous staining
Total	40	40	40	-	-	-

The result of this study showed that malaria parasites were stained with a modified solution of Giemsa dilutions of thick and thin blood smears, parasitic pigments could be distinguished on 15-20 min but we could not be read malaria parasites within time 25-30 min.

Table 3. The observation data of standard Giemsa staining to detection malarial parasites by microscopic as gold standard in Biak and Abepura General Hospitals

Turnaround time (min)	Standard Giemsa staining 3%	Parasite staining	Red blood cell staining	
		Thick/Thin	Thick	Thin
45 – 60	10 samples	parasite pigment distinct	complete hemolysis	Homogenous Staining
Turnaround time (min)	Standard Giemsa staining 10%	Parasite staining Thick/Thin	Red blood cell staining	
			Thick	Thin
15 – 20	10 samples	Parasite pigment distinct	complete hemolysis	Homogenous Staining
Turnaround time (min)	1:3	Parasite staining Thick/Thin	Red blood cell staining	
			Thick	Thin
25 – 30	10 samples	Parasite pigment	complete	Homogenous

		distinct	hemolysis	Staining

The result of this study shows that malaria parasites stained solution of standard Giemsa 3%.10% and 1:3 to thick and thin blood smears, parasitic pigments parasites could be read and differentiated between the nucleus and cytoplasm of the parasites.

Discussion

The result study showed that precise method of examining malaria preparations were using a modified of Giemsa staining 1:4 with pH7.2 and we need a time of 15 - 20 min to detect the number of malaria parasites and parasite image, it is clear that the nucleus is red and the cytoplasm is blue and gametocytes and there is Maurer and Shuffler's dots with used a electric binocular microscopic, the same as the standard Giemsa method 1:3 with a time of 30-45min and 3% with a time of 45-60 min and 10% with a time 10-15 min and a pH of 7.2, respectively, because the erythrocytes were completely lysed and the malaria parasites can be read properly. This result almost the same with [3,6]

The modified method of Giemsa is dilutions of 1:5 and 1:6 with a time of 5-10 min. The research shown that the malaria parasites was visible in the nucleus and cytoplasm but the amount of Plasmodium that was read through an electric binocular microscope was reduced because the concentration of Giemsa staining is too thick and caused the erythrocytes not to lysed completely and half malaria parasites covered by Giemsa when compared with Giemsa standard 1:3,3% and 10% with a H 7.2, respectively [3,7]. Our results suggest that the modified staining method of 1:5 and 1:6 Giemsa dilutions cannot be used for antimalarial drug failure test or the success of antimalarial drug in treating patients. .

To evaluation results of the modified Giemsa staining of 1:4 can be used because the observation show that the number of Plasmodium Parasites were found but the same as the observation for the Giemsa standard 1:3, 3% and 10% solutions, respectively, but the same observation was not founded in the modified Giemsa staining solutions of 1:5 and 1:6 because on the microscope image there is Giemsa particles were still settling and the nucleus and cytoplasm staining is present but not clear, erythrocytes are not completely lysed, the background of the preparation is soiled, the color of the cytoplasm becomes pale, the number of Plasmodium parasites were reduced and wasteful of Giemsa[3,9,11]

Conclusions

The result shown that the modified Giemsa 1:4 staining method is very good for detecting malaria parasites on thick and thin blood smears because the results of the semi-quantitative and quantitative count of the number of Plasmodium are the same as those from the same patients are using the standard Giemsa 1:3, 3% and 10%, respectively. And our result suggest that Giemsa staining modifications 1:5 and 1:6 cannot be used to detect malaria parasites because the concentrations of giemsa staining is too concentrated so that half of the erythrocytes are not completely lysed, the nucleus and cytoplasm are not clearly visible microscopically and the number malaria parasites were reduced because half of malaria parasites are covered by Giemsa.

Microscopic evaluation showed that there is still Giemsa particles that accumulate and nucleus and cytoplasmic staining of the parasites were not clearly visible, the erythrocytes were not completely lysed, the background of the preparations were soiled, the cytoplasmic was pale, the number of malaria parasites decreased and wasteful of Giemsa.

Abbreviation

Pf:Plasmodium falciparum, Pv: Plasmodium vivax, CI:Confidence Interval, Pv: Probability value

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Availability of data and materials

The data sets analyzed in this study are available from the corresponding author on request

Ethics approval and consent to patients

Ethical approval for this research was obtained from the ministry of health research ethic committee of the Polytechnic of health Jayapura.

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