ORIGINAL ARTICLE

New Methylene Blue Stain for Malaria Detection on Thin Smears

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Abstract:

Background: Malaria is the most important parasitic infection of man. Microscopy remains gold standard in malaria diagnosis. Management of malaria requires rapid detection of parasite in human blood. Hence there is a need to develop another diagnostic method with less limitation, which will address this issue. *Aim and Objectives:* To find a low cost reliable and accurate method for malaria detection on peripheral smear. *Material and Methods:* A prospective study of 40 cases was done. Two thin smears were prepared for each case; one was stained with Leishman stain and other with new methylene blue stain and examined under oil immersion. The smears were examined individually by two pathologists and results were prepared. Different parasitic morphologic forms were looked for. Parasitemia percentage was calculated. We also compared number of fields required to diagnose with both stains in positive cases. *Results*: In this study we found that 25 (83.3%) cases were detected in less than 50 fields using New Methylene blue stain against 18 (60.0%) cases with Leishman stain. We also found 100% sensitivity and specificity for New Methylene blue stain, whereas Leishman stain showed 90% sensitivity and specificity of 85%. *Conclusion*: The detection of malaria parasite was considerably easy with New Methylene Blue stain and required less time in comparison with Leishman stain.

Keywords: Malaria, New Methylene Blue Stain, Leishman stain, Parasitemia

Introduction:

Malaria is the most important mosquito borne parasitic infection of man caused by parasites of the genus *Plasmodium* [1]. Four species of malaria parasite infect human beings; they are *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale* and *Plasmodium malariae* [2]. It is a leading cause of morbidity and mortality worldwide. Malaria is prevalent in tropical and subtropical regions because of significant amount of rainfall, warm temperature, high humidity and stagnant water in which the larvae mature providing mosquitoes ideal environment needed for continuous breeding [1]. The symptoms of malaria such as fever, headache, malaise, fatigue and abdominal discomfort are very non specific which may lead to over treatment of malaria in endemic areas without laboratory diagnosis [3]. Early diagnosis of malaria is essential for malaria treatment and control. Microscopy remains gold standard in malaria diagnosis even though rapid diagnostic tests are increasingly used [4]. The advantage of microscopy is that it provides a quantitative assessment of peripheral blood parasitemia and parasite stages as well as information on other blood elements [5]. Diagnosis of malaria based on microscopy has central importance for species differentiation, parasitic quantification and

management of severe disease [2]. Microscopy requires technical expertise of the microscopist and is time consuming. To overcome the limitations of conventional microscopy many alternative rapid diagnostic non microscopic methods such as demonstration of acridine orange stained parasites in capillary centrifuged blood, rapid immuno-chromatographic assays and the sensitive molecular techniques like DNA hybridization and polymerase chain reaction have been introduced. These methods also have their own limitations and should be used as complementary method to conventional microscopy [6]. Management of malaria requires rapid detection of presence of parasite in human blood and early institutionalization of antimalarial drugs. According to WHO, all cases of suspected malaria should be confirmed by microscopy or rapid diagnostic tests before administering treatment [2]. Hence there is a need to develop another diagnostic method with less limitation, which will address this issue.

Material and Methods:

A prospective study of 40 cases including 30 cases positive for malaria and 10 cases negative for malaria diagnosed by Leishman stained smears, was done in haematopathology section of Department of Pathology, BLDE University's, Shri B. M. Patil Medical College, Vijayapura, Karnataka. All universal precautions were taken while preparing the smears. Two thin smears were prepared for each case; one was stained with Leishman stain and other with new methylene blue stain and examined under oil immersion.

The samples were divided into 3 groups based on the percentage of parasitemia. The smears by both the stains were examined individually by two pathologists and results were prepared. Smears were examined for different parasitic morphologic forms like trophozoites, schizonts and gametocytes. Also hemozoin pigment character was observed in the smears. Parasitemia percentage was calculated.

The smears were scanned carefully and minimum of 200 oil immersion fields were examined before labeling a slide as 'Negative for Malaria'. If only occasional infected red cells are seen then 'less than 1%' value was given. One negative smear makes diagnosis of malaria unlikely but in clinically suspected cases; smears were repeated every 6-12 hours for 48 hours to rule out malaria. Also presence of malarial pigment in circulating neutrophils and monocytes was looked for.

Results:

Malaria parasite can be detected by peripheral smear examination using Leishman stain and new methylene blue stain as in Table 1.

The percentage of parasitemia was calculated by calculating number of infected red blood cells per 1000 red cells and expressed as percentage (Table 2).

$$\text{Parasitemia \%} = \frac{\text{Number of infected red cells}}{\text{1000 RBCs}}$$

Table 1: Detection of Malaria Parasite by Peripheral Smear Examination using Leishman Stain and New Methylene Blue Stain

Method	Ring Stage of Malaria Parasite under Microscope		
	Nucleus	Cytoplasm	Pigment
New Methylene Blue Stain	Dark blue	-	Refractile brown-black
Leishman Stain	Red	Blue	Brown-black

Table 2: Distribution of Cases According to Parasitemia Percentage

Parasitemia Percentage	Number of Cases (Positive Cases = 30)
<2%	16
2-5%	10
5-10%	4

Table 3: Comparison between Positive Cases using Leishman Stain and New Methylene Blue Stain

Identified Positive Cases in Number of Fields	Leishman Stain (positive cases = 30)	New Methylene Blue Stain (positive cases = 30)
<50 Oil Immersion Fields	18	25
50-100 Oil Immersion Fields	8	5
100-200 Oil Immersion Fields	4	-

Table 4: Shows Sensitivity and Specificity of Leishman and New Methylene Blue stain

Stain	Sensitivity	Specificity
Leishman Stain	90%	85%
New Methylene Blue Stain	100%	100%

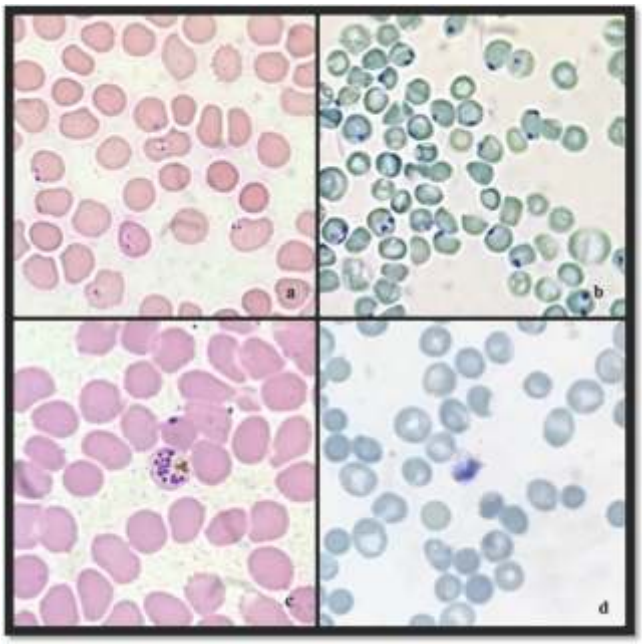

Fig. 1: a) Plasmodium Falciparum showing Two Rings on Leishman Stain b) Plasmodium Falciparum showing Two Chromatin Dots on New Methylene Blue Stain c) Schizonts of Plasmodium Vivax on Leishman Stain d) Schizonts of Plasmodium Vivax Showing Pigment and Nuclear Chromatin Dots on New Methylene Blue Stain

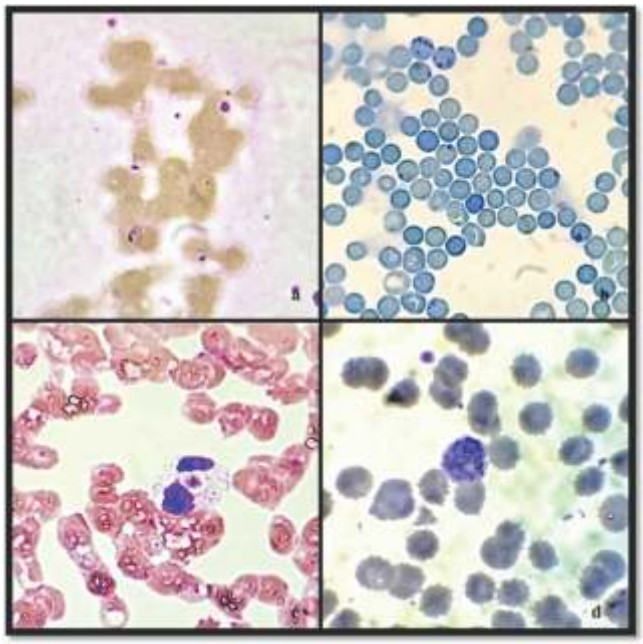

Fig. 2: a) Plasmodium Vivax showing One Ring on Leishman Stain b) Plasmodium Vivax showing One Chromatin Dot on New Methylene Blue Stain c) Engulfed Malaria Parasite within Neutrophil on Leishman Stain d) Basophilic Stippling within RBC which can be Confused with Malaria Parasite in Leishman Stain

Discussion:

This is a novel and one of its kind studies as here we have compared the number of oil immersion fields required for making the diagnosis. It is recommended that at least 200 oil immersion fields should be examined before labeling a peripheral smear negative for malaria. In a laboratory in the resource poor endemic zone, this has become cumbersome for pathologists to screen multiple cases stained with Leishman stain. Thick smears have a higher sensitivity of detection of 5-10 parasites/µl but due to red cell lysis, morphology and species identification is difficult, for which a second thin smear stained with Leishman stain needs to be done. Thick smear method is tedious with multiple steps. Thin smears stained with Leishman stain are used for species identification and have a sensitivity of 200 parasites/ µl. But if the single thin smear prepared, is stained with new methylene blue stain the sensitivity is maintained along with the morphology. This helps to reduce the turnaround time, reduces false negative and false positive cases and the sensitivity of the test is also maintained.

The identification of parasite was easier with new methylene blue stain, as only the various forms of parasites, reticulocytes and leukocytes take the dark blue stain against the background of pale blue to pale green color, giving high contrast to parasites. There were no artifacts seen as in Leishman stain which usually results in confusion, like the stain precipitates and red cell artifacts leading to increase in false positive and false negative test results. Also new methylene blue stain allowed species identification as it preserves the morphology unlike thick smears. The number of chromatin dots and nuclear features were better appreciated in new methylene blue stain than Leishman stain. It was observed that hemozoin pigment was better appreciated with new methylene blue stain than Leishman stain (Fig. 1 and 2).

In this study we found that 25 (83.3%) cases were detected in less than 50 fields using new methylene blue stain against 18 (60.0%) cases with Leishman stain. (Table 3) We found 100% sensitivity and specificity for new methylene blue stain, whereas Leishman stain showed 90% sensitivity and specificity of 85% because of false positive and false negative results due to artifacts (Table 4).

Lema *et al*. (1999) [6] found 98% sensitivity and 100% specificity for field stain which has new methylene blue as a component and 92% sensitivity and 85% specificity for Giemsa stain which is also a Romanowsky stain like Leishman stain. They also found that Giemsa staining is time consuming and is more suitable for batch staining rather than individual samples whereas Fields staining is quick and can be utilized for individual as well as batch staining. Occasional washing off of the blood film was the disadvantage with fields stain.

Iqbal *et al*. (2003) [7] in their comparative study between Giemsa and modified Giemsa stain found that modified Giemsa stain was better at diagnosing with better nuclear and pigment staining characteristics of malarial parasites. They found that the turnaround time was drastically reduced from 45-60 minutes to 15-20 minutes with the use of modified Giemsa stain over the standard Giemsa stain. The hemolysis in thick films was better with modified Giemsa stain than standard Giemsa stain. Also species identification was possible in thick films in up to 65% cases with Modified Giemsa stain than 20% cases with

standard Giemsa stain. Occasional washing off of the blood films was noted with Modified Giemsa stain.

Mohapatra *et al*. (2014) [8] found that new methylene blue stain (in 90% cases) detected Gametocytes of *Plasmodium vivax* better than Giemsa stain (in 83% cases). They also detected gametocyte count 2-3 times higher in new methylene blue stained smears than Giemsa stained smears. Hemozoin pigment was better appreciated with new methylene blue stain. Like our study they also found that average parasitemia was nearly equal by both the stains, suggesting that both stains are equally efficient in detecting the asexual forms of parasite.

Conclusion:

The detection of malaria parasite was considerably easy with New Methylene Blue stain and required less time due to good contrast properties of the stain. The pigment character was also better appreciated by new methylene stain in comparison with Leishman stain.

Thus, in present study it was found that the peripheral thin smears with New Methylene blue stain is better and easier method for detection of malaria parasite than with the Leishman stain. Hence it can be used in place of Leishman stain in suspected cases of malaria.

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