Significance of Fluorescence Microscopy in the Diagnosis of Malaria

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(Received: December, 2014)          (Accepted: January, 2015)

ABSTRACT:
The present study was undertaken to find out a new easy and comparable method with the Gold standard in the diagnosis of malaria. Fluorescence microscopy with stained smear was found to be a feasible and more sensitive procedure. Blood samples were collected from 256 patients of suspected malaria & were subjected to all three diagnostic modalities—peripheral blood smear (PS), fluorescent microscopy with stained smear (FM), and antigen detection test using Plasmodium lactate dehydrogenase (pLDH) and aldolase (AG). The results of various methods were compared. It was seen that fluorescence microscopy (FM) when compared with PS (Thick and thin smears) the sensitivity & specificity was found to be 92.4% & 77.4% respectively. FM when compared with Antigen was found to be 94.9% & 92.3% respectively. FM gives sensitivity close to AG which is the most sensitive test & the specificity is better which is close to PS which is gold standard. FM is as sensitive as AG test and as specific as PS in species identification. It is a Ultra sensitive and feasible procedure too.

KEY WORDS: fluorescence microscopy; gold standard, malaria;

INTRODUCTION:
Malaria is one of the major human infectious diseases in over 100 endemic countries with approximately 300 million clinical cases and 2 million fatalities per year.[1,3] Prompt and accurate diagnosis is one of the key for effective disease management, being one of the main interventions of the global malaria control strategy.[2,4] Conventional light microscopy is widely used for the detection and quantification of malaria parasites, and is recognized as the gold standard. In most settings, the procedure consists of: collecting a finger-prick blood sample; preparing thin and thick blood smears; staining the smears with Giemsa; and examining the smears under a microscope for the presence of malaria parasites in the erythrocytes.[5] However, this microscopic detection method is exacting and depends on a good staining technique and well supervised technicians. Milne et al[6] found that most routine diagnostic laboratories generally achieved low detection sensitivity (average, 0.01% parasitemia) on examination of the results from British laboratories submitted to the Malaria Reference Laboratory. Even with excellent erythrocyte preparation and good technicians, the detection limit is low (0.001% parasitemia) and approximately 1 hour is required for the detection of a sufficient number of infected erythrocytes.[7,8] So, it is quite difficult to detect malaria infection before the appearance of severe symptoms including high fever. Although immunochromatography was recently developed for malaria detection with easy operation and a rapid detection time (20 min), the detection limit is similar to that of microscopic observation with Giemsa staining.[9,10] Although several new methods of malaria diagnosis based on flow cytometry or real-time Polymerase Chain Reaction (PCR) have been developed[11,12] some disadvantages remain, i.e. being costly and the relatively low detection limit for flow cytometry and the requirement of several hours for the detection of malaria parasites by real-time PCR. For prevention of the spread of malaria in the world, it is necessary to develop an easy, sensitive, accurate and convenient diagnosis system.[3]
Recently, microchip technologies have developed a single-cell microarray for the analysis of antigen-specific single B-cells to detect the secretion of antibodies by single cells and analysis with a microarray scanner for detection of the presence of fluorescence-positive nuclei in erythrocytes.[13,14]

The potential of a cell microarray chip system for the early diagnosis of malaria was found to be ultra-highly sensitive and allowed accurate detection of malaria-infected erythrocytes but these methods are costly, time consuming & require skilled trained individuals. But the Fluorescence microscopy study is simple, cost effective, requires untrained non skilled individual. It is highly sensitive & very specific, as good as “Gold Standard”-light microscopy, but is feasible and easy to perform.

MATERIALS AND METHODS:

The study was conducted in central Pathology laboratory of NKP Salve Institute of Medical Sciences after obtaining clearance from the Institutional Ethics Committee. Blood samples were collected from 256 patients attending the OPD and IPD with clinical suspicion of malaria, in one year period. Approximately 2 ml of venous blood was collected from each patient.

Sample processing: Firstly, thick and thin smears were prepared as per the standard method described and the smears were stained with Leishman stain. The average time spent on screening each slide varied depending on parasite density. Thick smears were reported negative after examination of 200–300 oil immersion fields with no parasite. A thin smear was given negative when no parasites were observed in 200 oil immersion fields. Secondly, for fluorescence microscopy sets of slides were prepared and stained by flooding with Auramine for 15 min then rinsed by Distilled Water (DW). Decolourised for 30-60 seconds by flooding fluorescence decoloriser after rinsing again with DW counter stained by potassium permanganate for 2 min, rinsed with DW and air dried. Finally the slides were placed in a closed box to prevent exposure to light. All slides stained with Leishman and Auramine Orange (AO) were examined under light microscope and fluorescence microscope respectively. The projecting images were obtained which were modified for color brightness and contrast using adobe photo shop to facilitate printing. Thirdly, antigen detection using pLDH and aldolase commercially available antigen detection kit detecting plasmodium LDH and aldolase were used. The test was conducted using anticoagulated venous blood. The sample was added to test strip using a calibrated dropper provided with the kit, and the strip was placed in a micro well containing buffer. The result was read after 15 min as per manufacturer’s instructions. It was interpreted as positive for \( P. falciparum \) if T1 and control (C) bands were seen. If only T2 and C bands were seen it was interpreted as positive for \( P. vivax \). If T1, T2 and C bands were seen it was indicative of mixed infection.

The data was analyzed statistically for specificity, sensitivity, predictive values and accuracy.

RESULTS:

The three diagnostic modalities gave varied results as shown in Table 1. The incidence of malaria was found to be 64.06% (164/256). Table 2 shows that the PS was positive in 136 cases out of which 39 (28.67%) were positive for \( P. falciparum \), 93 for \( P. vivax \) and 4 samples were positive for mixed infection. Florescence microscopy showed 146 positive for malaria of which 41 cases were positive for \( P. falciparum \), 98 for \( P. vivax \) and 7 for mixed infection. The antigen was positive in 155 cases with 43 being positive for \( P. falciparum \), 105 for \( P. vivax \) and 7 for mixed infection. Table 3 shows the specificity and sensitivity of PS v FM and FM vs AG. We used the PS test as gold standard to compare our results and calculate the sensitivity and specificity of methods used by us. Florescence as compared to PS (Gold Standard) had a sensitivity and specificity of 92.4% and 77.4% respectively, while FM vs. antigen showed 94% and 92.3% respectively for detection of malaria. AG showed sensitivity of 94.9% and specificity of 92.3%. Hence FM showed the specificity sensitivity close to AG. Table 3 also shows the positive and negative predictive values and accuracy of various methods.

DISCUSSION:

In the present study we compared three methods for the detection of malaria, to find an easy but sensitive and cost-effective method. We found the FM, using Florescence stain to be as sensitive as antigen and as specific as the PS technique for the diagnosis of malaria. Florescence microscopy was compared to conventional smear (PS) technique, more cases were detected. It is cheap, easy to perform and cost-effective. The simple equipment required for this technique is available even in peripheral laboratories. Hence, it can also be used for malaria diagnosis at the
Florescence microscopy when compared to the antigen test showed a sensitivity of 94.9% and specificity of 92.3%, with a positive predictive value of 94.62 and negative predictive value of 92.5% (Table 3). The PS and Florescence negative patients were detected by antigen test. The four cases were benefitted greatly by undergoing the antigen test at the same time. However, two cases which were negative on PS were missed by the antigen test, but picked up on florescence microscopy. This can be explained as insufficient enzyme production, which occurs during early malarial infection, or if the patient’s blood samples contained parasites at concentration below the optimal test detection level (50–100 parasite/
ml of blood). Thus, the antigen test is of importance only in PS negative cases and could be preferably used as a final diagnostic test and not as a screening test or first line of investigation considering its high cost. The use of FM (Digital microscope) in the rural parts of our country is difficult[8], Fluorescence microscopy (Portable) could easily be done even in the rural areas, although PS is considered to be the gold standard.[9]

However, more such studies would be required to establish this as a good screening test for malaria.

**CONCLUSION:**

Our study proved that in the trial of the various diagnostic modalities in malaria, Fluorescence microscopy and antigen detection were found superior to PS. Our new simple method i.e. Fluorescence eliminates the false positive results of antigen. It is also easy to perform and cost-effective. FM is simple, easy, more sensitive, more specific, cost effective (except digital microscope) easily available, and can be performed by non skilled individual too. The test can be done any where i.e. remote places, for epidemic survey, or can be used as an economical diagnostic test in the diagnosis of malaria. Fluorescence portable microscope can be established as a new gold standard in the diagnosis of malaria.

**REFERENCES:**