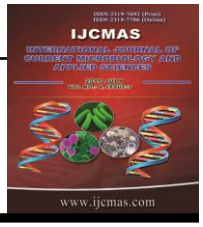
Int.J.Curr.Microbiol.App.Sci (2015) 4(8): 832-839

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 8 (2015) pp. 832-839**
http://www.ijcmas.com



Original Research Article

Fluorescence Based Methods for Rapid Diagnosis of Malaria

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Keywords

Acridine Orange, Malaria, Quantitative Buffy Coat, Blood smear, Navi Mumbai

A B S T R A C T

Malaria is associated with high morbidity and mortality in tropical and sub-tropical countries. The aim of this study was to establish the fluorescent microscopy based method for diagnosis of malaria. 100 blood samples were collected in Ethylene diaminetetra acetic acid (EDTA) Vacutainer tube from clinically suspected malaria patients. Each sample was processed as a) thick and thin smear stained with Leishman's stain for light microscopic examination, b) Acridine orange stain and fluorescent microscopy and c) Quantitative Buffy Coat test according to the kit procedure. Detection of malarial parasites by light microscopy, Acridine Orange stain and Fluorescent microscopy and Quantitative Buffy Coat test was 13%, 16% and 20% respectively. There is a need to standardize the fluorescent based methods for detection of malarial parasites

Introduction

Malaria imposes great socio-economic burden on humanity and one of the highest killer diseases affecting most tropical countries. More than 2 billion people (40% of the world's population) live in areas where malaria is endemic. It was estimated that over 250 million people worldwide contracted malaria in 2002 (Doderer *et al.*, 2007). According to the UNICEF, in every minute, malaria kills a child in the world (UNICEF-Health-Malaria). Of all the human malaria parasites, *Plasmodium falciparum* (*P. falciparum*) is most pathogenic and frequently fatal if not treated in time (UNICEF-Health-Malaria). In India, according to Nandwani *et al.* (2005) a total of 1.82 million cases of malaria and 0.89 million cases of *P. falciparum* cases were reported in the year 2002. According to National Vector Borne Disease Control Programme (NVBDCP, 2001–12) there were 10,66,981 malaria positive cases and 5,33,535 *P. falciparum* in the year 2012.

The increasing incidence of falciparum malaria, the need to identify and treat the additional infective carriers (reservoirs) and to reduce the chances of transmission has given an impetus for development of simple and rapid methods for the diagnosis of falciparum malaria. Conventional Leishman's, Giemsa or Romanowsky's

stained peripheral blood examination by light microscopy is the standard method for malaria diagnosis in malaria endemic countries. Conventional light microscopy has the advantages that it is relatively inexpensive, provides permanent record and can be shared with other disease control programmes. However, it suffers from disadvantages such as it is labour intensive and time consuming (Mendiratta *et al.*, 2006).

The purpose of this study was to establish the fluorescent based methods for early detection of malarial parasites.

Materials and methods

This prospective study was carried out at Department of Microbiology, MGM Medical College and Hospital, Kamothe, Navi Mumbai, India, over a period of one year from July 2014 to December 2014. Chi-square test, Z tests and SPSS (version 17) software was used for statistical analysis. A total of 100 samples collected from clinically suspected cases of malaria of all the age groups in both the sexes attending tertiary care hospital were included for study. Incase of MP smear negative – patients with other positive lab test results – for typhoid fever and dengue fever were excluded from the study.

Ethical clearance: Ethical clearance was obtained from the Institutional Ethical committee of MGM Institute of Health Sciences (Deemed University), Navi Mumbai before starting the project.

Sample collection: The detailed history, clinical signs and symptoms were recorded in the proforma. 3–5 ml venous blood was collected into Ethylene diamine tetra acetic acid (EDTA) tube (Becton Dickinson) under sterile precautions. Standard thick and thin smears were prepared.

The smears were stained with Leishman's stain (Lot No. 0000168288-HiMedia Laboratories Pvt. Ltd., India) and observed under 100x oil immersion objective lens. The blood collected in EDTA was subjected to Acridine orange (AO) and Quantitative Buffy Coat (QBC) test (Fig. 1 & 2).

Thick and thin smears were prepared on a clean grease free glass slide. Thick smears dehaemoglobinized and stained with Leishman's stain according to standard procedure.

Acridine orange staining

The acridine orange stain (Sigma, USA) was prepared by dissolving 50 mg of the dye in 4 ml glacial acetic acid and diluting to 200 ml with distilled water to make working stain and kept in amber coloured bottle. The stain was stored at 4°C for more than 6 months. 1-2 drops of blood sample from EDTA tube was mixed with 1 drop of working acridine orange stain over a glass slide, covered with coverslip and examined under Fluorescent microscope (Nikon –Eclipse). The AO stained smears scanned at 10x and the suspected parasitic structure confirmed under 40x or under 100x oil immersion lens.

Quantitative buffy coat test

Quantitative Buffy Coat test was done using Quantitative Buffy Coat kit (Diagnour RFCL Ltd., India). The Quantitative Buffy Coat malaria tube was filled with venous blood up to black marking keeping tube nearly horizontal, rolled tube between fingers several times to mix blood and anticoagulants. Tube was rolled between fingers at least 10 times or for at least 5 seconds to mix blood with coating of Acridine Orange. Tube was sealed using tube plug and then tube suspender inserted into open end of Quantitative Buffy Coat

tube using clean forceps, provided with the kit. QBC tube was centrifuged immediately. QBC tube placed on rotor of micro centrifuge and centrifuged at 12000 RPM for 5 minutes.

Results and Discussion

Total 100 blood samples were taken from malaria suspected patients after obtaining informed consent. 13 samples out of 100 were positive for malarial parasites i.e. 13% by light microscopy, 16 samples out of 100 were positive for malarial parasites i.e. 16% by Acridine Orange stain and Fluorescent microscopy and 20 samples out of 100 were positive for malarial parasites i.e. 20% by Quantitative Buffy Coat test. Actual number of species detected by 3 methods is shown in Figure 5 and table 2.

In our study microscopic findings showed *Plasmodium vivax* 53.85%, *Plasmodium falciparum* 15.38% and mixed species 30.77% (Fig. 6).

Acridine orange showed *Plasmodium vivax* 56.25%, *Plasmodium falciparum* 25% and mixed species 18.75% (Fig. 7).

The Quantitative Buffy Coat test showed *Plasmodium vivax* 50%, *Plasmodium falciparum* 35% and mixed species 15% (Fig. 8).

Table.1 Showing comparison of different methods for diagnosis of malarial parasites

Methods	**Malaria positive**	**Percentages**
Microscopy	13/100	13%
Acridine orange	16/100	16%
QBC test	20/100	20%

Table.2 Showing comparison of diagnostic methods for detection of malarial parasites

Methods	***P. vivax***	***P. falciparum***	**Mixed species**	**Total samples**
Microscopy	7 (53.85%)	2 (15.38%)	4 (30.77%)	13 (100%)
Acridine Orange stain	9 (56.25%)	4 (25%)	3 (18.75%)	16 (100%)
QBC test	10 (50%)	7 (35%)	3 (15%)	20 (100%)
Chi-square = 2.10, df = 4, P value > 0.05, statistically not significant.				

Figure.1 Showing malarial parasites in Acridine orange stain

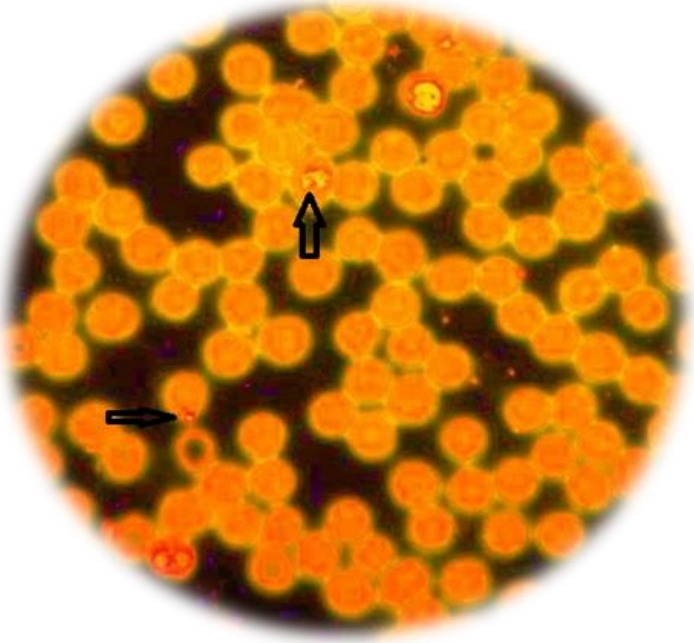


Figure.2 Showing malarial parasites in QBC test

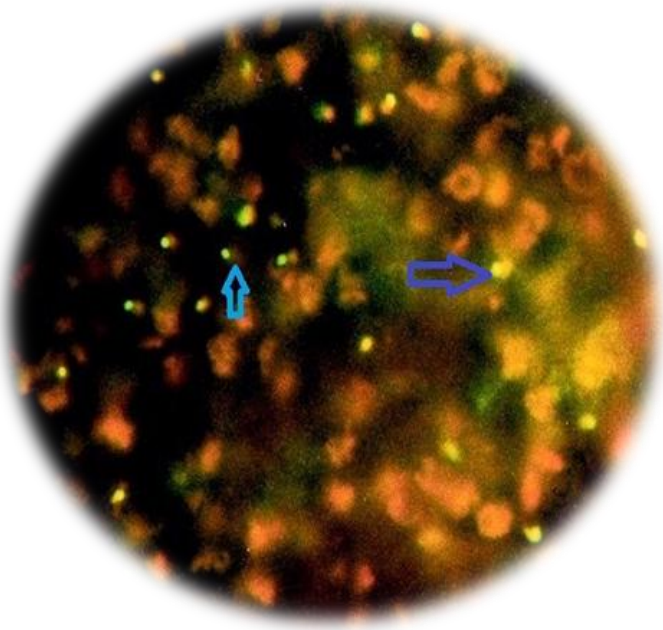


Figure.3 Showing sensitivity of various methods for detection of malarial parasites

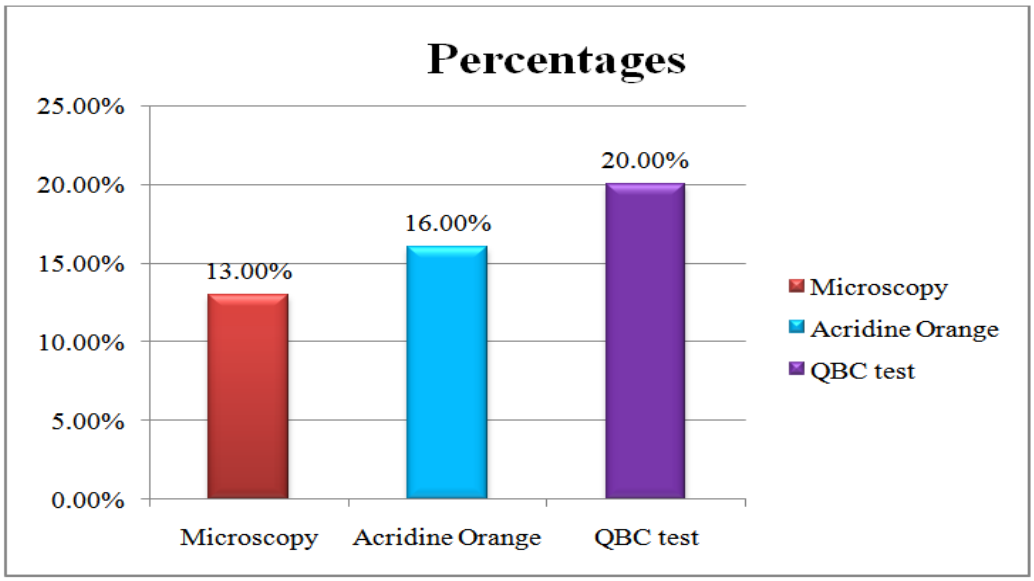
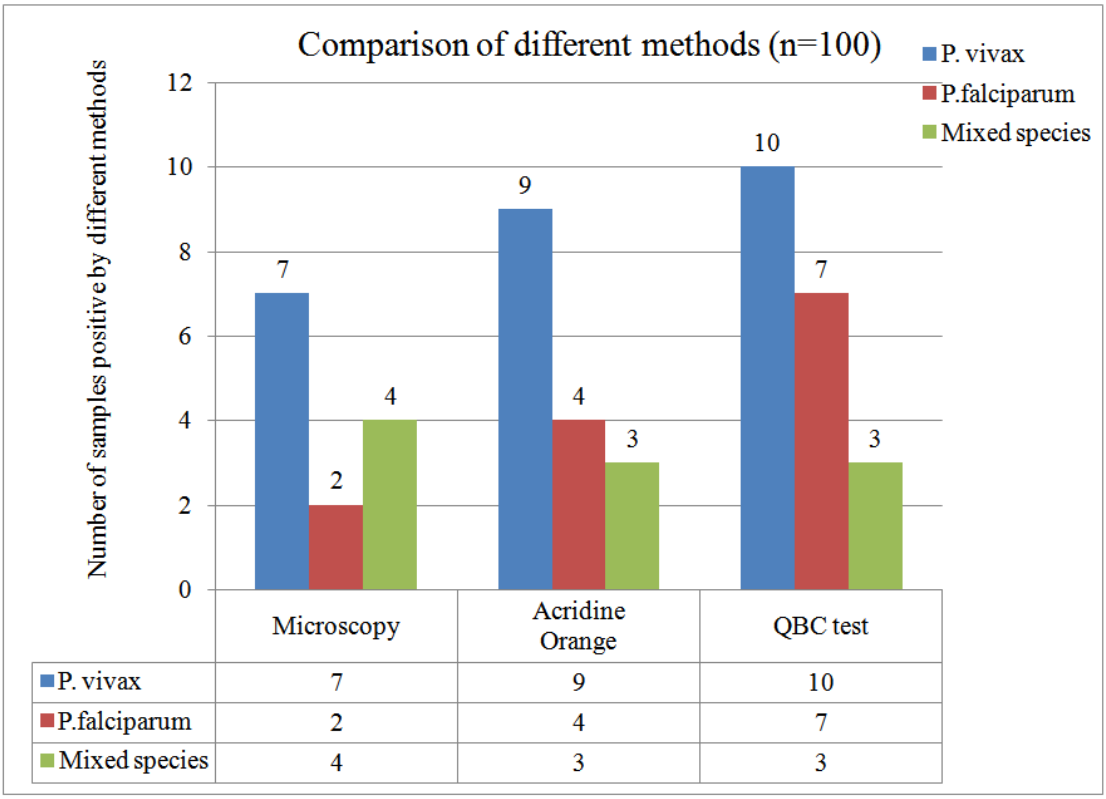


Figure.4 Showing specificity of various methods for detection of malarial parasites



	Microscopy	Acridine Orange	QBC test
P. vivax	7	9	10
P.falciparum	2	4	7
Mixed species	4	3	3

Figure.5 Showing species characterization of malarial parasites by microscopy

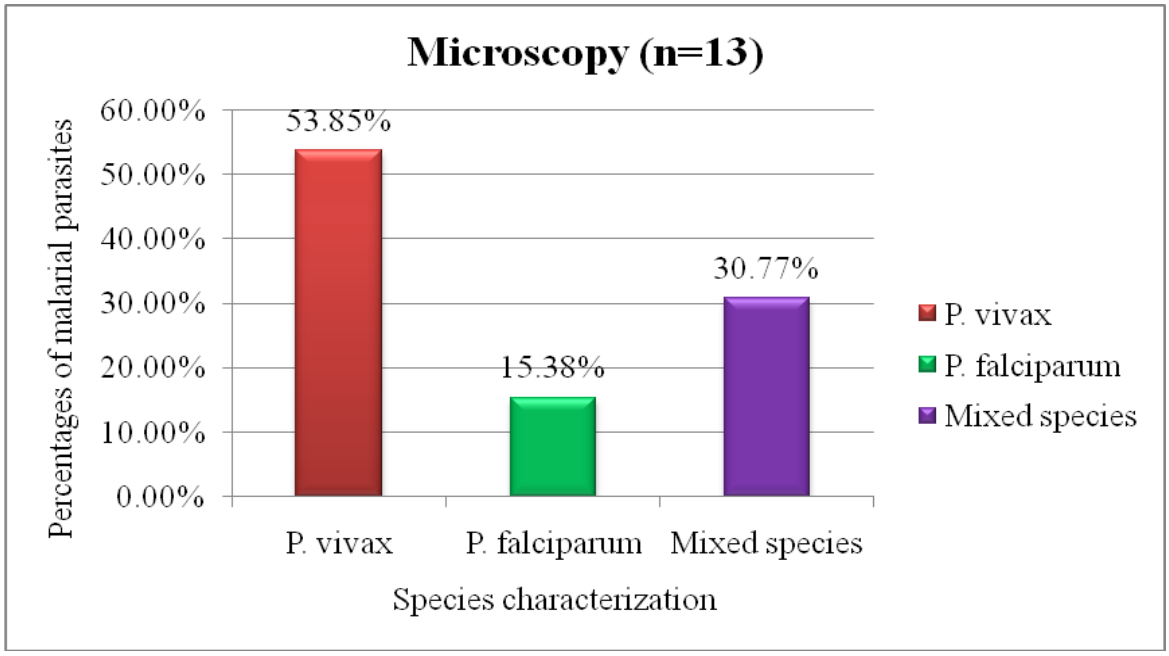


Figure.6 Showing species characterization of malarial parasites by Acridine Orange stain and Fluorescent microscopy

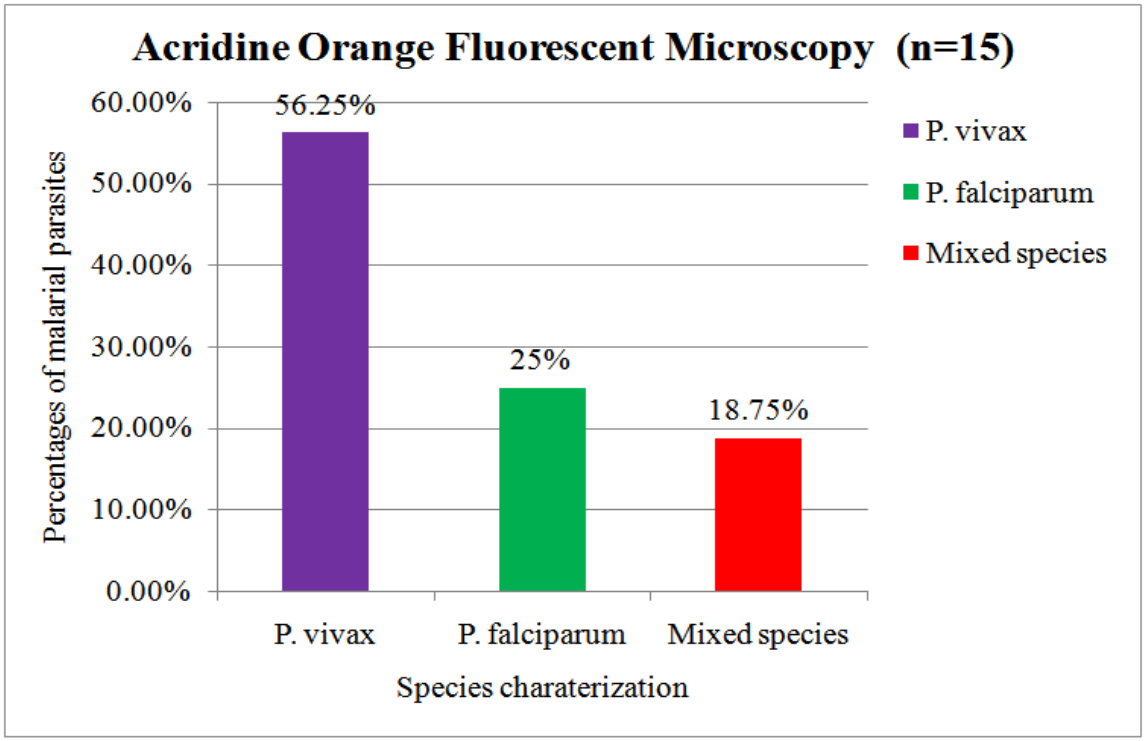
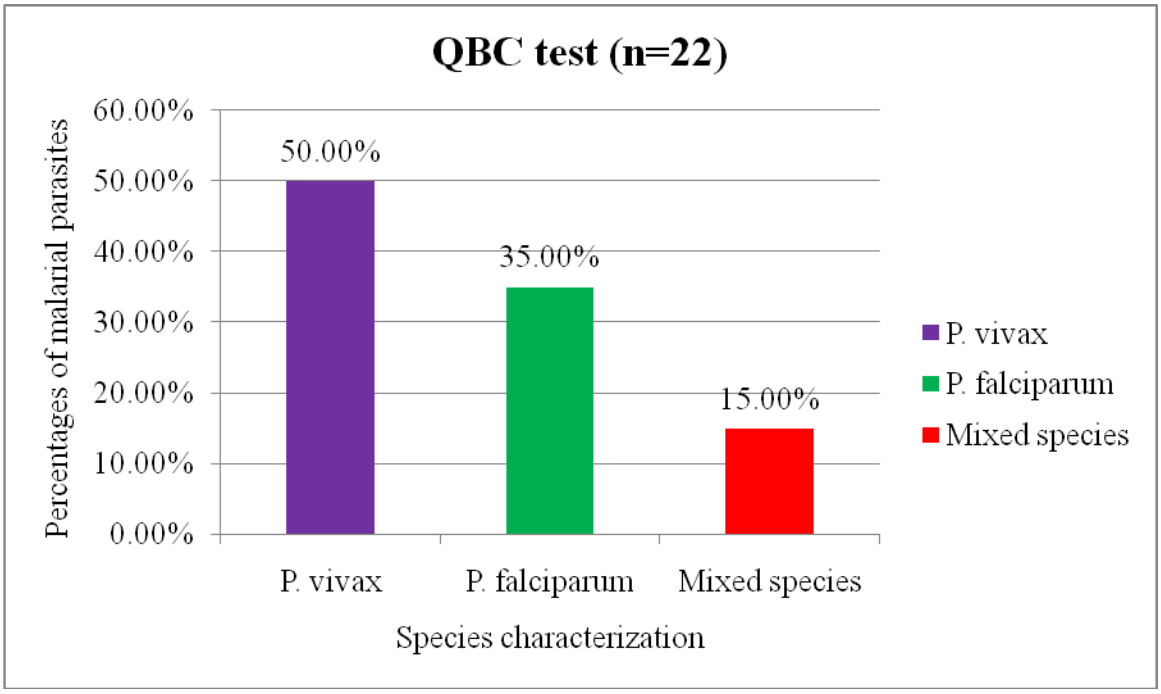


Figure.7 Showing species characterization of malarial parasites by QBC test



The present study was undertaken for standardize the fluorescent based method for detection of malarial parasites. Many workers reported on malaria diagnosis in India and other countries, but still in India maximum places malaria examined by light microscopy and they did not use the new techniques which has great impact over the light microscopy and there are need of using newer techniques instead of old one. The severity of malaria disease is depending on geographic location, disease burden, endemicity and the methods followed by the specialist.

All over world routine diagnosis of malaria is made by examination of blood films stained by Field / Giemsa / Leishman's stain using light microscope. This method is time consuming and open to subjective variation, leading to false negative or false positive results. Hence there is a need of more specific and rapid method of diagnosis of malaria. The present study was undertaken to standardize fluorescence based microscopic examination for diagnosis of malaria.

100 blood samples of suspected malaria cases were studied by different methods. Light microscopy, Acridine orange stain by Fluorescent microscope and QBC test showed positivity of malarial parasites as 13%, 16% and 20% respectively in our study (Table 1). In our study microscopic findings showed *Plasmodium vivax* 53.85%, *Plasmodium falciparum* 15.38% and mixed species 30.77%. Acridine orange showed *Plasmodium vivax* 56.25%, *Plasmodium falciparum* 25% and mixed species 18.75%. The Quantitative Buffy Coat test showed *Plasmodium vivax* 50%, *Plasmodium falciparum* 35% and mixed species 15%.

In our study microscopy findings (13%) compared well with DK Mendiratta et al.[5] (18.28%) and Aparna Y et al.[6] (13.87%). Positivity of malarial parasites by Acridine orange stain in our study (16%) correlated with DK Mendiratta et al.[5] (18.28%).

Our results of QBC test (20%) correlate well with Aparna Y et al.[6] (20.44%).

Comparison of these three methods clearly shows advantages of Fluorescence microscopic examination (Acridine orange stain and QBC test) over light microscopic examination. These fluorescence methods also detected more number of *P. falciparum* cases which are associated with high morbidity and mortality. QBC test is best method but requires special capillary tubes, high speed micro-centrifuge machine and trained personnel to carry out procedure and microscopic examination both.

Acridine orange Fluorescence staining method is simple, easy to perform and requires only Fluorescence microscope as special equipment. Routine use of this method will help detect more number of malaria cases and proper treatment.

Acknowledgements

We are thankful to Dr. S.N. Kadam, Hon'ble Vice Chancellor and Dr. Chander P. Puri, Hon'ble Pro Vice Chancellor (Research), MGM Institute of Health Sciences (Deemed University), Navi Mumbai for providing Fluorescence Microscope. We also thank Dr. Rajiv R. Rao Consultant Pathologist at Dr. Jairaj's Diagnostic Centre, CBD Belapur, Navi Mumbai, India for providing micro centrifuge machine and QBC test equipments (Para Lens Advanced w/60x objective and ParaViewer). Also we acknowledge Mr. Pandurang Thatkar (Statistician) for his help during data analysis.

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