

Diagnostic methods of malaria in Eastern Nepal: a comparative study of traditional and two rapid diagnostic tests

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ABSTRACT

This study compared the diagnostic accuracy of Quantitative Buffy Coat (QBC[®]) a fluorescent microscopy test and OptiMAL[®], an immunochromatographic dip stick test against conventional microscopy for the detection of malaria at a tertiary teaching hospital situated in Eastern Nepal. 100 clinically suspected malaria patients with positive and negative parasitemia were assessed under conventional microscopy. The blood samples withdrawn from these subjects were further evaluated by the QBC Method and OptiMAL dipstick test. The sensitivity, specificity, positive predictive value, negative predictive value and diagnostic accuracy of QBC and OptiMAL tests as compared with microscopy were 100%, 100%, 100%, 100%, 1 and 96%, 100%, 100%, 96.15% and 0.98 respectively. In Nepal, thick and thin blood smears remain the gold standard for malaria species diagnosis in routine diagnostic laboratories. In this study the efficacy of newer malaria rapid diagnostic tests (RDT) surpassed the diagnostic efficacy of clinical microscopy and hence these RDT's will have a greater role in clinical practice. The cost of QBC technique may impose limitations on its use in Nepal but the OptiMAL test is likely to play an important part in urgent malaria diagnosis.

Keywords: Malaria, Microscopy, QBC[®], OptiMAL[®], Parasitemia, Rapid Diagnostic Tests (RDT)

INTRODUCTION

Malaria is one of the most serious diseases to affect people in the developing countries with tropical and subtropical climates.¹ Worldwide, approximately 2 billion individuals are at risk; 100 million develop overt clinical disease and 1.5-2.7 million die every year.² More than 70% of the total population of Nepal is at risk of malaria. A high incidence of malaria was observed in hilly and mountainous districts (Kavre, Sindhupalchowk, Bhojpur and Dhankutta) as well as in Terai districts (Kanchanpur and Bardiya) that belong to the forest related eco-epidemiological type of malaria. Focal outbreaks of malaria during the months of August-September of 1999-2000 were reported from these districts and *Plasmodium falciparum* was the predominant strain detected (65% of the cases). In Sunsari district, malaria parasite incidence rate per thousand populations is 0.04%, and blood slide examination positivity is 0.6%.³

One of the most pronounced problems in controlling the morbidity and mortality caused by malaria is limited access to active diagnosis and treatment in areas where malaria is endemic.⁴ Currently, the access to effective diagnosis is limited in much of the developing world; where microscopy remains the gold standard and most cost-effective method.⁴⁻⁹ Examination of Giemsa stained thick and thin blood smears can be problematic, since it

is labor-intensive and it requires considerable expertise for its interpretation, particularly at low levels of parasitemia.^{6,8,10} The availability of rapid, simple, and specific diagnostic tools will assist in the control of malaria by allowing therapy to be accurately and aggressively administered. The present study compared the diagnostic accuracy of newer rapid malaria tests (RDT) Quantitative Buffy Coat (QBC) a fluorescent microscopy test and OptiMAL, an immunochromatographic dip stick test against conventional microscopy the gold standard, for the detection of malaria at a tertiary teaching hospital situated in Eastern Nepal.

MATERIALS AND METHODS

All clinically suspected malaria patients, who attended BP Koirala institute of health Sciences (BPKIHS) from April 2004 to March 2005, were included in the study. The blood samples were drawn from these patients at the general out-patient department and emergency department, before the administration of antimalarials. Samples were evaluated by Giemsa stained thick and thin film microscopy, the Quantitative Buffy Coat (QBC) method and OptiMAL dipstick test. Patients who had taken antimalarial drugs before collection of blood sample were not included in the study.

Microscopy: Thick and thin smears were prepared on

Table -1: Performances of QBC and OptiMAL with peripheral blood smear examination for malaria parasite detection

Test Results		Gold Standard (Giemsa stain)		Total	Se (%)	Sp (%)	PPV (%)	NPV (%)	DA
		Positive	Negative						
QBC	Positive	50	0	50	100	100	100	100	1
	Negative	0	50	50					
	Total	50	50	100					
OptiMAL	Positive	48	0	48	96	100	100	96.15	0.98
	Negative	2	50	52					
	Total	50	50	100					

Se: Sensitivity, **Sp:** Specificity, **PPV:** Positive Predictive Value, **NPV:** Negative Predictive Value, **DA:** Diagnostic Accuracy.

the same slide and Giemsa stained as per standard laboratory protocol. Under microscope, the parasites were easily detected in the thick film. The young trophozoites appeared as incomplete rings or blue cytoplasm with a detached red chromatin dot. In the late trophozoites of *Plasmodium vivax*, the fragmented cytoplasm was seen. Schizonts and gametocytes of *Plasmodium vivax* and *Plasmodium falciparum* were seen. In the thin films, the basic characters of the stained malarial parasite inside the RBC were demonstrated – cytoplasm stained blue and chromatin stained red. The presence of stippling on the red cell i.e., Schuffner’s dot was seen in *P. vivax*. Crescent shape gametocytes were found in *Plasmodium falciparum* and round gametocytes were found in *Plasmodium vivax*.

Determination of parasitemia: The numbers of parasites per 200 white blood cells (WBC) were counted in thick film smear. If the number of parasites were less than 10, then this was counted in respect to 500 WBC. Taking WBC count as 8000/mm³ of blood (normal value) the parasite count would be:

No. of parasite counted X 8000 = parasite/ µl of blood.

No. of WBC counted

Table-2: Parasite load ascertained by peripheral blood smear examination in the study

Parasite Range (per µl)	n ^s	Mean (per µl)	Std. Deviation	Minimum	Maximum	F	Sig.
1,000-10,000	14	4,914	3,048	1,160	9,920	72.35	0.000*
10,040-100,000	31	42,203	24,953	10,080	94,040		
>100,040	3	171,640	37,205	140,400	212,800		
Total	48 ^s	39,417	43,984	1,160	212,800		

*ANOVA: p<0.001 highly significant. ^s2 subjects having only gametocytes are excluded.

During the examination of thick and thin blood films, if only gametocytes were seen; then the slides were reported as the species but parasite load was not reported.

QBC method: Microhematocrit centrifugation with the use of QBC malaria tube was used for the detection of blood parasite as per the manufacturer’s instructions.¹¹ The QBC tubes-a high precision glass hematocrit tube, pre-coated internally with Acridine orange stain and Potassium oxalate were filled with 55-65 µl of blood. A clear plastic closure was attached and a

precisely made cylindrical float, designed to be suspended in the packed red blood cells was inserted. The tube was centrifuged at 12,000 rotations per minute for 5 minutes. The components of the Buffy coat separate according to the densities, forming discrete bands. After centrifugation, the QBC tube was placed on a microscope tube holder and examined using a standard light microscope equipped with a UV Microscope adapter. Fluorescing parasites were then observed at the red blood cell - white blood cell interface.

Interpretation of results was done as follows:

- + -1-10 parasites per 100 thick film fields.
- ++ -11-100 parasites per 100 thick film fields.
- +++ -1-10 parasites per 1 thick film fields.
- ++++ -more than 10 parasites per 1thick film field.

OptiMAL dipstick test: OptiMAL dipstick is based on parasite Lactate Dehydrogenase (pLDH) which is actively produced by all human malaria parasite species during their growth in red cells. A series of monoclonal and polyclonal antibodies incorporated into the dipstick form was used to detect pLDH. Differentiation of malaria species in the OptiMAL test is based on antigen differences between pLDH isoforms. The OptiMAL

conjugate well contains an indicator-tagged monoclonal (mouse hybridoma) antibody to pLDH-dried on its surface. This monoclonal antibody reacts with all isoenzymes-LDHs of the genus Plasmodium. As per the package insert,¹² one drop of buffer was dispensed into the conjugate well and 4 drops into the wash well and after 1 minute, one drop of blood (~10µl of blood) was added in the conjugate well. The dipstick was placed

vertically into the conjugate well and was allowed to stand for 10 minutes. The blood migrated towards the filter pads and the control band appeared progressively. The dipstick was transferred into the conjugate well and left until the reaction field of the dipstick was cleared. When the control band became weakly visible (5-10 min) the result was read as follows:

Only one red line - negative

Two red lines - *Plasmodium vivax* (Pv), *Plasmodium malariae* (Pm), *Plasmodium ovale* (Po)

Three red lines - *Plasmodium falciparum* (pf) or mixed infections (pf and other plasmodium species)

Statistical analysis of the collected data was done using SPSS version 11.5

RESULTS

This study included a total of 100 subjects who had suspected clinical malaria. 65 subjects were men and 35 were women. The age range was 05-87 years with a mean of 31.48 (SD±18.12) years. Out of these 100 subjects, 50 were malaria positive cases and 50 were controls (negative cases). The sensitivity, specificity, positive predictive value, negative predictive value and diagnostic accuracy of QBC and OptiMAL tests as compared with microscopy were 100%, 100%, 100%, 100%, 1 and 96%, 100%, 100%, 96.15% and 0.98 respectively (Table-1).

P. falciparum was found in 64% (n=32), *P. vivax* in 30% (n=15) and 6% (n=3) mixed infections by OptiMAL test. OptiMAL test was negative in 2 subjects suffering from *P. falciparum* malaria (trophozoite) who were positive by microscopy. The mean parasite load of malaria positive subjects (n=48) was $39,417 \pm 43,984/\mu\text{l}$ of blood with a range of 1,160 to 212,800. Two subjects had only gametocytes detected in their blood sample (Table-2). Significantly a high number of patients, i.e. 34 cases (71%) reporting to this hospital had severe parasitemia i.e., parasitemia above 10,000/ μl of blood (Table-3).

Table-3: Comparison of Parasite load of malaria species ascertained by peripheral blood smear examination

Parasite range (per μl)	Parasite species % (n)			Total
	<i>P. falciparum</i>	<i>P. vivax</i>	Mixed Infections	
1,000-10,000	20% (6)	40% (6)	67% (2)	29% (14)
10,040-100,000	70% (21)	60% (9)	33% (1)	65% (31)
>100,040	10% (3)	0% (0)	0% (0)	6% (3)
Total	100% (30)	100% (15)	100%(3)	100%(48*)

Chi-Square Tests: Pearson Chi-Square = 5.353, p=0.253 (Not significant). *2 subjects having only gametocytes were excluded.

DISCUSSION

The results of the present hospital-based investigation are encouraging. The QBC and OptiMAL tests permitted accurate diagnosis within a short period of time. The results of these newer diagnostic tests in this study were, well above the WHO requirements for the rapid diagnostic tests.⁵

The QBC method is more sensitive, rapid, and practical than thick blood film for the diagnosis of malaria.¹³ The main concerns of this test are cost and the need for special equipment (centrifuge, fluorescence microscope, and capillary tubes). These features are clearly undesirable for fieldwork conditions.^{7,14} A study conducted in 2001, reported a sensitivity of 91.7% and a specificity of 88.9%, with the QBC assay when compared with microscopy.¹³ In general, the QBC results are corroborated by previous studies being in the same range.¹⁵⁻¹⁸ In our study it was 100% sensitive and specific probably because of the high parasite load, and there were no false identification. The discrimination of *Plasmodium* species by the QBC assay poses some difficulties because the discrimination of *P. vivax* from *P. falciparum* is very difficult in some of the patients; hence identification of the species by QBC was not attempted in our study.

Ease of use, convenience in the clinical setting, and low cost are important considerations for the selection of diagnostic laboratory tests.^{19,20} The cost of QBC test when compared to OptiMAL is much lower and hence it is being used routinely at this center. The availability of a good quality microscope with a built-in power illumination and technical expertise are essential for good-quality microscopy. However owing to the scarcity of these two factors in this part of the world, especially in field settings, alternative methods of malarial diagnosis hold a lot of promise. The most promising antigen detection methods for malaria diagnosis are the immunochromatographic dipstick tests. These tests are based on the immunologic detection of parasite lactate dehydrogenase and histidine rich protein-2 (HRP-2). A

field trial conducted in Kanchanpur and Dhanusha districts of Nepal reported a sensitivity of 97% and specificity of 98% for OptiMAL test.²¹ The results of present study are comparable to this study with a sensitivity of 96% and specificity of 100%. It is expected for OptiMAL test to achieve better results in cases with high parasitemias, as most studies worldwide have reported, decreased sensitivity of OptiMAL test when the parasite density is <100 parasites/ μl , and sensitivity and

specificity ranging above 95% when parasitemia exceeds 1000 parasites/ μl .^{5,7,8,14,22-28}

A parasite count is a useful indicator of disease severity, which might be very high in case of *P. falciparum*. In patients with imported malaria (non-immunes) the appearance of any parasite is clinically significant. However, in endemic areas, where individuals have a certain immunity, asymptomatic carriage is common and parasitemias of 5,000–10,000/ μl are generally regarded as the pyrogenic threshold.²³ The mean parasite load and range have not been reported from Nepal, however in a recent study of 153 cases, 54 cases were reported to be having a parasitemia load of more than 10,004 parasites/ μl of blood during an epidemic at Guthiparasauni village of Western Nepal.²⁹ The parasite densities reported in our study were above 1,000 parasites/ μl for all species of malaria. Seventy-one percent of all the malaria positive cases had parasite densities greater than, 10,040 parasites/ μl . As a tertiary care referral centre sample, it is reasonable to expect, cases with high parasitemia or recurrent malaria to be present in the study sample.

A serious point of concern in this study is the two false negative cases seen with OptiMAL test in *P. falciparum* and both cases had more than, 10,000 parasites/ μl (36,160 and 14,120 parasites/ μl). The occurrence of false-negative dipstick test results at higher parasitemia levels has been noted by others.^{24,27,30} It is not clear why this happens, and this issue has to be addressed by the manufacturers and researchers in the coming years. The possibility of gene deletion isolates that do not express HRP-2 has been postulated¹, although the same evidence for pLDH has not been discovered. Further investigations for these and other non-expressed antigens should be considered.¹⁴ Until these issues are addressed, we agree and recommend microscopic examination of peripheral smears to be done in all suspected malaria cases as backup (for definitive diagnosis), although OptiMAL can serve as a screening method in the case of emergency and field situations where, beginning chemotherapy for malaria will be urgent and essential.

The failure of OptiMAL dipstick to detect cases with only gametocytes has been well documented in many studies.^{8,9,26} Possible explanations include the presence of blocking antibodies or immune complex formation and an absence of pLDH production by gametocytes.³¹ But in this study there were two falciparum cases with only gametocytes, which were detected by OptiMAL. Similarly, Moody has reported detection of 5 cases with gametocytes-only by OptiMAL.¹⁴

Identification of different malaria parasites is important, as treatment varies according to the species type.

Plasmodium falciparum infections can be fatal and in the case of *P. vivax* and *P. ovale*, hypnozoites remain dormant in liver cells and can cause relapse.¹³ The detection of gametocytes can mean one of several things. In untreated patients they indicate active infection, while in partially treated patients, they may indicate persistent infection.³² They can also occur after successful treatment.^{13,26,33} The present study reports only 32 cases of *P. falciparum* malaria (64%) and 15 cases of *P. vivax* malaria (30%), with 3 cases (6%) of mixed infections with the former being the predominant species. Studies conducted previously in this region have reported *P. vivax* to be the predominant species (up to 85%) and *P. falciparum* infections (7-19%) emerging rapidly.^{3,21,29,33} Studies from neighboring country have reported similar trends.^{26,33} Countries with imported malaria differ with regard to which species predominates. One centre in Canada reported 50% *P. vivax* and 40% *P. falciparum* infections,³⁴ but another study reported 51% *P. falciparum* and 40% *P. vivax* infections in the UK,³⁵ while 67% *P. falciparum* and 20% *P. vivax* were reported from Germany.³⁶ The data from our study shows a trend of high, *P. falciparum* cases. This high trend of *P. falciparum* among the patients in this group might be influenced by high severity/relapse and hence reported to this tertiary care referral center in Eastern Nepal. Furthermore, a majority of the blood samples collected were from the emergency laboratory may explain the preponderance of high *P. falciparum* cases in this study.

Several of the rapid tests that have been developed for the detection of malarial infection, such as the ParaSight F[®] test¹¹, the ICT Malaria Pf Test[®] and the Determine[®] test are based on the detection of histidine rich protein-2 (HRP-2). As this antigen is only released from erythrocytes infected with *P. falciparum*, such tests cannot be used to detect infections with *P. vivax*.²⁶ Furthermore, HRP-2 antigen remains detectable several days after the completion of drug treatment even in a parasitemic patients,^{26,37,38} this makes it difficult for health care providers to assess accurately the effectiveness of drug therapy. The ICT Malaria Pf/Pv test can detect both *P. vivax* and *P. falciparum* infections but its sensitivity in the detection of *P. vivax* (72%–75%) is relatively low.²⁶ The capacity of the OptiMAL test to diagnose *P. falciparum* and *P. vivax* infections with high specificity and sensitivity is of great clinical utility, particularly where the two species are co-endemic and the drug of choice is different for the two.³¹

In this study mixed infections were detected in three cases. The configuration of the strip and detecting antibodies used ensure that a pure infection with *P. vivax* would show one line plus the control line, while two

lines plus the control line could indicate a pure infection with *P. falciparum* or a mixed infection with *P. falciparum* plus other *Plasmodium* species.^{7,9,12,14} Although mixed infections are uncommon, it would be desirable for mixed infections containing *P. falciparum* always to be read as that species rather than another species, given the more serious nature of *P. falciparum* infections.⁸ The ability of the OptiMAL test to distinguish pure *P. vivax* infections from pure *P. falciparum* or mixed *P. falciparum/P. vivax/Plasmodium* species infections may increase its use.⁸

Sensitive, rapid diagnostic tests for malaria that can be read easily by individuals with minimal training in a rural clinic setting, or during the night in hospitals, remote from diagnostic laboratories, are highly desirable. The cost of the tests will clearly influence the extent to which they are deployed in any setting, but would be offset by reducing the morbidity and expenditure on unnecessary treatment that occur when an inaccurate diagnosis is made. The World Health Organization⁵ is in need of rapid and precise diagnostic tools to assist in controlling malaria and to limit the associated mortality and morbidity. The present data demonstrate that the OptiMAL test may find use in hospitals, in the efficient management of acute and complicated malaria cases and, under field conditions, in determining the drug-resistance profiles of the local parasites. The OptiMAL test may prove to be a reliable alternative to microscopy for the diagnosis of *P. falciparum* or *P. vivax* infection, especially in resource-poor areas like ours. However, if the OptiMAL test is ever to be used routinely in malaria-control programs in developing countries, it must be made cheaper. Whatever the OptiMAL test costs, the cost-effectiveness of the accurate diagnosis of malaria will become increasingly apparent as relatively cheap drugs such as Chloroquine and Sulfadoxine-Pyrimethamine become ineffective.⁸

We anticipate that in Nepal, the OptiMAL test is likely to play an important part in urgent malaria diagnosis at night and at weekends, when routine laboratories are closed and when relatively inexperienced microscopists may be on duty and also in screening programs. However, Giemsa stained peripheral blood smear will still remain the backup diagnostic aid till further improvements occur with the immunochromatographic tests.

ACKNOWLEDGEMENTS

The authors would like to thank: Prof. Dr. Samuel Bhattacharya, Professor and Head, Prof. T. M. Mohapatra, formerly Professor and Head, Prof. Dr. Basudha Khanal, and Dr. Rajendra Gurung of Department of Microbiology, Prof. Prahlad Karki, Head, Department of Internal Medicine, Prof.

Mary Lewis and Prof. Owen Lewis from the Department of Family Medicine, BPKIHS, Dharan. Prof. Dr. Deepali Roy Choudhary, Professor and Head and Dr. Ritu Amatya, Associate professor, Department of Microbiology, NMCTH, Kathmandu.

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