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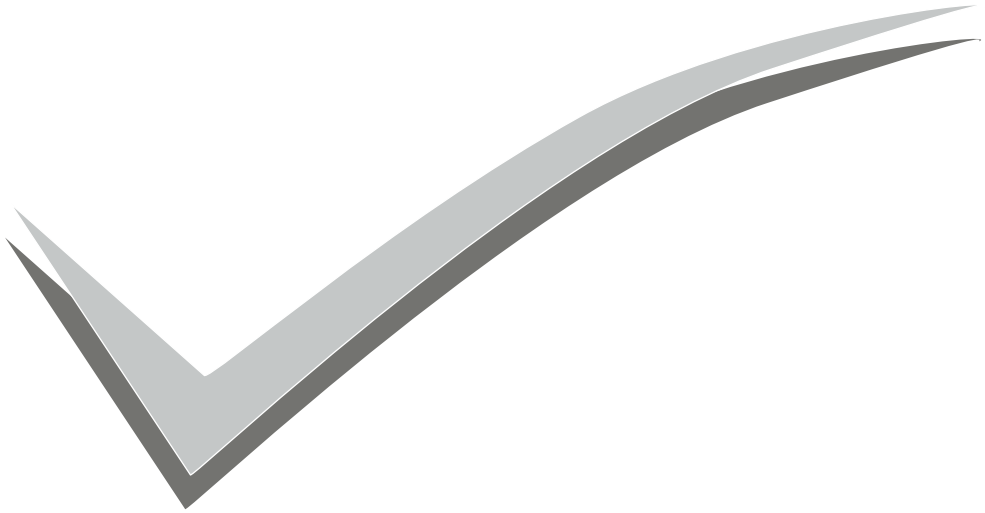

FALCIVAX®

Rapid test for Malaria (Pv/Pf)

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INDEX

S. No.	Name of the Publication	Pg Nos
1.	Journal of Evolution of Medical and Dental Sciences, Vol.2, Issue 7, Feb.2013	712-714
2.	Journal of Bacteriology and Parasitology, Vol.2 , Issue 8, 1000125	1-3
3.	Malaria Journal, 2011, 10:175	1-9
4.	Elsevier, The Royal Society of Tropical Medicine and Hygiene (2009)	1-8
5.	Plos One, May 2014, Vol 9, Issue 5, e98442	1-10
6.	Malaria Journal, 2011, 10:367	1-7
7.	Malaria Journal, 2009,8:173	1-11
8.	Asian Pacific Journal of Tropical Medicine (2012)	79-82
9.	Asian Pacific Journal of Tropical BioMedicine (2014)	S81-S86
10.	Journal of Vector Borne Disease 50, December(2013)	318-320
11.	Acta Parasitologica, 2010, 55,1	96-98
12.	International Journal of Emergency Medicine 2012, 5,11	1-5

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EXTERNAL EVALUATIONS

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S. No.	**Name of the Evaluating Body**
13.	WHO Malaria RDT Testing Round 4
14.	ICMR, Goa, 2003

Reference Articles on Google Books

- Staining V/s Immunological Techniques for Diagnosis of Malaria.

"A COMPARATIVE EVALUATION OF CONVENTIONAL STAINING METHODS AND IMMUNOLOGICAL TECHNIQUES FOR THE DIAGNOSIS OF MALARIA"

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AIMS AND OBJECTIVES: To compare the conventional staining methods (Leishman stain, JSB stain, field's stain) on peripheral blood smear with rapid immunological methods (Falcivax (HRP2Ag+ pLDH) & SD Bioline (pLDH)) for the diagnosis of malarial parasites and to achieve at a better diagnostic tool for diagnosis of malarial parasites in terms of cost, technical expertise, lab facilities, rapidity, higher sensitivity and specificity. **MATERIALS AND METHODS:** The present study was conducted in the Microbiology Department of a MGM Medical College & Hospital , Navi Mumbai from July 2009 to June 2010. 339 patients with clinical features s/o Malaria were included in the study. For all the patients in the study group, 1ml of blood was collected by venipuncture aseptically in EDTA bulbs. Thick and thin blood films were prepared on 3 slides one each for Leishman, Fields and JSB stain. All the samples were then subjected to antigen detection using Falcivax (Pf & Pv) and SD Bioline (Pf & Pv) Kits. Test was performed as per kit instruction manual and interpreted accordingly. **RESULTS:** A total of 339 cases were studied, 238 males and 101 females with age ranges from 15yrs to 72yrs. Of 339 cases, 138 cases were positive for malarial parasites by gold standard viz: Leishman stained thick smear. 68 cases were infected with P.falciparum, 51 with P.vivax and 19 had mixed infection. Falcivax kit showed higher sensitivity and specificity compared to rest of all the rapid methods for detection of P.falciparum. In diagnosing P.falciparum malaria it showed sensitivity of 100% in comparison with gold standard even at low parasitemias. In fact, Falcivax kits also detect falciparum infections even when the parasites are sequestered and not detectable by microscopy. In case of detection of P.vivax, performance of SD bioline and Falcivax kits were similar (96%) and better than conventional staining of thin blood smear examination (92%) by Leishman, JSB and Field's method when compared with gold standard thick smear examination.
CONCLUSION: The logistic, economic and technical factors limit rapid access to microscopic confirmation of malaria in many tropical countries including India. So taking into account of all the factors from our study, we recommend Falcivax HRP2 antigen detection as a tool for diagnosis of malaria in future. Although no test can completely replace the conventional method of peripheral blood smear examination, these newer diagnostic tests can be used as supplement to microscopic examination of peripheral blood smear where the diagnosis cannot be made on microscopy alone. Also, rapid immunochromatography tests can be used in times of urgency

and in areas where facilities of microscopy are not available especially during night times when services of laboratory and experienced Microscopists are not available.

INTRODUCTION: Malaria is the most important parasitic disease of mankind and known since antiquity. The human disease is a protozoan infection of red blood cells transmitted by the bite of a blood feeding female anopheline mosquito[2]. Prevalence of malaria is around 300 million people worldwide with a global death rate over 1.5 million/year. [1,2].

Malaria is a serious disease caused by the protozoal parasite Plasmodium, and if left untreated, can be fatal. Only four of the known species of Plasmodium are able to infect humans: P. falciparum, P. vivax, P.ovale, and P. malariae. The symptoms of malaria include high fevers, chills, rigors, and flu-like illness. Because the symptoms are so familiar, they are often misdiagnosed. The increasing incidence of malaria, the need to identify and treat the additional carriers (reservoirs) and to reduce the chances of transmission has given an impetus for development of simple and rapid methods for the diagnosis of malaria. Conventional Leishman or Giemsa stained peripheral blood smear examination remain the gold standard for the diagnosis of malaria in malaria endemic countries. However it has disadvantages such as it is labor-intensive, time consuming and requires expertise. [4]

Numerous rapid techniques like acridine orange(AO) stain, Quantitative Buffy coat (QBC), detection of soluble histidine rich protein II antigen (HRP2 Ag), pLDH and aldolase enzymes in whole blood have been evaluated to diagnose malaria. Malaria presents a diagnostic challenge to laboratories in most countries. This study attempts to review the current methodology and approach to the diagnosis of malaria in a practical and helpful way for the laboratory and for the physician caring for the patient. The urgency and importance of obtaining results quickly from the examination of blood samples from patients with suspected acute malaria render some of the more sensitive methods like PCR, DNA Probe assay for malaria diagnosis impractical for routine laboratory use, although they may be considered reference procedures. [3,4,5] So, in the present study an attempt is made to evaluate and compare maximum number of rapid, feasible and economical tests for diagnosis of malaria with the Leishman stained thick blood smears which is considered as Gold standard. These tests include JSB stain, Field's stain and rapid immunological techniques viz; Falcivax (HRP2Ag+ pLDH) & SD bioline (pLDH).

Keeping in mind the seriousness of condition and the current availability of diagnostic modalities, the present study was carried out in a tertiary care hospital in Navi Mumbai with the following aims and objectives 1) To study the comparative efficacy of the peripheral smear examination by Leishman stain, JSB stain, field's stain and rapid immunologically techniques viz; Falcivax (HRP2Ag+ pLDH) & SD Bioline (pLDH) for diagnosis of Malaria. 2) To determine the most optimum diagnostic tool for diagnosis of malarial parasites in terms of cost, technical expertise, lab facilities, rapidity, higher sensitivity and specificity.

MATERIALS AND METHODS:METHODOLOGY: The present study was conducted in the Microbiology Department of a tertiary Care Hospital from July 2009 to June 2010. The study was cleared by Institutional ethics committee. 339 patients were included in the study.

CRITERIA FOR ENROLLING PATIENTS

INCLUSION CRITERIA:

Patients with clinical features s/o Malaria which includes

- history of fever within the preceding 48 hrs,
- chills and rigors, or
- fever at the time of presentation or
- Fever of unknown origin, with or without splenomegaly, systemic complications or CNS manifestations.

EXCLUSION CRITERIA:

- Those patients treated for malaria in last 2 weeks.
- Those patients with confirmed diagnosis of infections like typhoid, pneumonia, Urinary tract infection, Septicemia, dengue, or leptospirosis were ruled out from the study.

SAMPLE COLLECTION: For all the patients in the study group, 1ml of blood is collected by venipuncture aseptically in EDTA bulbs. Ethics committee and Research committee of the institution had approved the study

CONVENTIONAL STAINING METHODS:

SAMPLE PREPARATION (THIN FILM AND THICK FILM): For diagnosis of malaria, thick and thin smears are prepared from blood collected in EDTA. Thick and thin blood films are prepared on 3 slides one each for Leishman, fields and JSB stain. Thick smear helps in rapid diagnosis, even when the parasitaemia is low. Thin smear is preferred for determination of species and morphological details of the parasite. Thin film also provides information regarding erythrocyte morphology, leukocytes, and platelets.

The thick smear was stained with Leishman stain. Thin smears were stained by all the three staining methods, Leishman stain, JSB stain and Field's Stain as per standard staining techniques.

METHODS OF COUNTING MALARIAL PARASITES IN THICK BLOOD FILMS

EXAMINATION OF THE THICK FILM

- At least 100 good fields are screened before a slide can be pronounced negative.
- Determination of parasites/µl of blood is done by enumerating the number of parasites in relation to a standard number of leukocytes/µl (8000). Initially smear is examined for the presence of parasites and species identification. Tally counter is used one for counting leucocytes and other for parasites. After counting 200 leucocytes number of parasites is recorded. Then a simple mathematical formula multiplying the number of parasites by 8000 then dividing this figure by 200(no. of leucocytes counted) is used.

Parasites counted/ no. of leucocytes x 8000 = parasites/µl of blood.

In this study, 200 leucocytes are counted, thus the formula:

No. of parasites x 40

EXAMINATION OF THIN FILM : examine for at least l00 fields to determine whether the blood film is positive or negative for malaria.

RAPID IMMUNOLOGICAL TECHNIQUES: All the samples are then subjected to antigen detection using Falcivax (Pf & Pv) and SD Bioline (Pf & Pv) Kits. Test was performed as per kit instruction manual and interpreted accordingly.

SD MALARIA ANTIGEN: TEST EXPLANATION & PRINCIPLE: The SD Malaria antigen test contains a membrane strip, which is precoated with two polyclonal antibodies as two separate lines across a test strip. One polyclonal antibodies (test line Pf) are specific to lactate dehydrogenase of P. falciparum and the other polyclonal antibodies (test line P.v/ pan) are Pan specific to the lactate dehydrogenase of plasmodium species.

FALCIVAX RAPID TEST FOR MALARIA (DEVICE): Falcivax is a rapid self performing, qualitative, two site sandwich immunoassay utilizing whole blood for the detection of P. falciparum specific histidine rich protein – 2 (HRP-2) and P. vivax specific pLDH. The test can also be used for specific detection and differentiation of P. falciparum and P. vivax malaria in areas with high rate of mixed infections.

TEST PROCEDURE – Were performed as per kit literature All the results of conventional staining methods and rapid immunological techniques are compared and evaluated for the sensitivity and specificity for the diagnosis of malaria.

RESULTS AND OBSERVATIONS: Of the 339 patients, 68 (20%) were positive for falciparum malaria by Leishman stained thick blood smear, 60(17%) by Leishman stained thin blood smear, 60(17%) by JSB stained thin smear, 60 (17%) by Field's stained thin smear, 69(20.3%) by Falcivax antigen Kit and 57(16.8%) by SD Bioline antigen Kit. Thin smear stained by Leishman, JSB and Fields gave similar results. They failed to detect 8 (11.8%) of the positives by Gold standard, while SD Bioline method failed to detect 11(16%) cases. Falcivax detected all the positive cases, but also detected one positive that was negative by Leishman stained Thick smear (gold Standard). All those positives by other methods were also positive by Gold standard.

Of the 339 patients, 51(15%) were positive for vivax malaria by Gold Standard Leishman stained thick blood smear, 47(13.8%) by Leishman stained thin blood smear, 47(13.8%) by JSB stained thin smear, 47(13.8%) by Field's stained thin smear, 49(14.4%) by Falcivax antigen Kit and 49(14.4%) by SD Bioline antigen Kit. thin smear stained by Leishman, JSB and Fields gave similar results. They failed to detect 4 (7%) of the positives by Gold standard, while SD Bioline and Falcivax methods failed to detect 2(3.9%) cases. All those positives detected by other methods were also positive by Gold standard.

There were 19 positive cases for mixed malarial parasite infection. All the methods under study gave same results for mixed infection as compared to gold standard.

Parasitic index was calculated for all the positive malaria cases by gold standard Leishman stained thick smear. The parasitemias ranged from 80-9600 parasites/µl. Sensitivity of all the rapid conventional staining methods was 100% at parasitemias > 200 parasites/µl for P.falciparum and 240 parasites/µl for P.vivax. But sensitivity was lowered to 88% and 92% for P.falciparum and P.vivax respectively with parasitemias <200parasites/µl. SD Bioline Antigen detection method had a detection threshold of 280parasites/µl for P.vivax and 200parasites/µl. Sensitivity of Falcivax Kit for P.falciparum was 100% at 80 parasites/µl. The detection limit for P.vivax was 200 parasites/µl. similar to that of SD Bioline, as for P.vivax, method of detection of vivax specific pLDH is same in both kits.

DATA ANALYSIS: Sensitivity, specificity, positive predictive value, negative predictive value were calculated for each method by comparing the proportion of positive and negative results for each method with the gold standard Leishman stained thick blood smear examination.

Prevalence = No. of positives by Leishman stained thick blood smear examination/ total no. of samples examined.

Sensitivity = No. of matching Positives by both Leishman stained thick smear examination(Gold std) and the method to be evaluated/no. of positives by Leishman stained thick blood smear examination. OR TP/TP+FN. This is no . of True Positives

Specificity = No. of matching negatives by both Leishman stained thick smear examination(Gold std) and the method to be evaluated/no. of positives by Leishman stained thick blood smear examination. OR TN/TN=FN. This is no. of True negatives.

Positive Predictive value (PPV) = sensitivity x (1-Prevalence)/ (sensitivity x prevalence) + (1-specificity) x (1- prevalence) OR TP/ TP + FP

Negative predictive value (NPV) = specificity x (1-Prevalence)/ specificity x (1- prevalence) + (1- sensitivity) x prevalence OR TN/FN+TN

The concordance of tests was calculated as follows ;

Concordance = No. of matching Positives and negatives by both Leishman stained thick smear examination(Gold std) and the method to be evaluated/ total no. of samples examined OR TP+ TN/ Total no. of samples examined.

The values obtained are multiplied by 100 and reported as percentage.

Where, TP-True positive, TN – True negative

With reference to the data shown in Table 10:

Prevalence of malaria = 40%

DISCUSSION: The majority of cases of malaria worldwide are treated on the basis of clinical diagnosis and microscopy. Several studies have shown that the ability to diagnose malaria by blood film examination alone is about 75% for P.falciparum.[6] Rapid detection and effective treatment of malaria is a prerequisite in reducing the morbidity and mortality due to the disease. Leishman stained thick smear examination, which is the corner stone in the laboratory diagnosis of malaria, has undergone little improvement since its inception. In the present study, We included 339 patients attending hospital over a period of one year for different complaints suggestive of malaria. We evaluated and compared five different techniques of rapid detection of malarial parasites with the Gold standard Leishman stained thick smear.

TYPE OF MALARIA: In India 40% of cases are as result of P.vivax malarial infection. 44.3% are due to P.falciparum malarial infection. 10-15% is due to mixed malarial infection.[1] In this study, P.falciparum contributed to 49% of the total study group, P.vivax contributed to 37% of the total study group and mixed malarial infection was responsible for the remaining of 14% of the study group.

Mendiratta et al[6] studied 443 cases with maximum number of P.falciparum cases. Thus our study compares well with WHO report and other similar studies regarding the increased incidence of P.falciparum.

In the present study, while comparing the available different methods of rapid detection for P.falciparum with gold standard Leishman stained thick blood smear microscopy, the sensitivity of Falcivax antigen kit was highest (100%) followed by Leishman stained thin smear (88%), JSB stain (88%), Fields stain (88%), and lastly by SD Bioline (83 %). For detection of P.

vivax, Both antigen Kits Falcivax and SD bioline (96%) detected highest no. of cases followed by thin peripheral smear microscopy by Leishman, JSB and Field's stain (92%). our results were in agreement with other studies.[5,6,7] In our study there were no false positive results by any of the methods suggesting 100% specificity, except Falcivax kit which showed one false positive for falciparum.

In the present study the results obtained by thin smear examination by Leishman stain, JSB stain and Fields stain were same. They had lower sensitivity of 88% for P.falciparum and 92 % for P.vivax. It was found that at lower parasitemia, the sensitivity dropped considerably. Sensitivity was 100% at Parasitemia level 240parasites/µl or more. Thick smear provides enhanced sensitivity of blood film technique and is much better than the thin film for detection of low levels of parasitemia and relapse or recrudescence[9]. Warhurst and Williams reported that examination of thin blood films is only 1/10 as sensitive as examination of thick blood films for the quantification of malarial parasites, although morphological identification of the Plasmodium species present is much easier using thin films. It is highly recommended that both thick and thin films be prepared and examined each time blood film examination for parasites is requested. [8]

Sensitivity of SD Bioline antigen kit, based on pLDH for P.falciparum was found to be lowest. The recommended method and current gold standard used for the routine laboratory diagnosis of malaria is the microscopic examination of stained thick blood films. In the most capable hands, this method can be expected to detect 50 parasites/µl (0.001% parasitemia) and to identify to the species level 98% of all parasites seen. In our study parasitemia level of 80parasites/µl or more were detected by thick smear microscopy. This procedure is recognized as difficult and time-consuming, requiring considerable training to obtain the necessary skills.
Immunochromatographic dipsticks offer the possibility of more rapid, nonmicroscopic methods for malaria diagnosis, thereby saving on training and time. These tests are easy to perform and require little training to interpret the results. On June 13, 2007, the U.S. Food and Drug Administration (FDA) approved the first RDT for use in the United States. This RDT is approved for use by hospital and commercial laboratories, not by individual clinicians or by patients themselves. It is recommended that all RDTs are followed-up with microscopy to confirm the results and if positive, to quantify the proportion of red blood cells that are infected.[10]

RDT currently on the market are easy to use; most are in cassette format with single-application areas for the blood sample and clearing buffer. We have also used Falcivax Kit which is in cassette format which is easier to perform as compared to SD bioline dipstick method. Most evaluation trials have included temperature and time stability for at least 1 year at 40°C.

The new generation of RDT offers a realistic practical chance to move the diagnosis of malaria away from the laboratory and nearer to the patient. our results with Falcivax Kit which is HRP2 based showed 100% sensitivity even at 80 parasites/µl as compared to pLDH based SD Bioline Kit and peripheral blood smear microscopy for diagnosis of P. falciparum. The added assurance that life-threatening parasitemia with P. falciparum will not be missed is welcome, particularly for inexperienced laboratory staff during night calls. The ability to detect the majority of the non-falciparum malaria cases also makes these tests ideally suited as major backup procedures for malaria diagnosis.

There are many considerations to be taken into account when reviewing the methods for laboratory diagnosis of malaria (Table 6), not the least of which are the important factors of availability and cost. The present debate on the introduction of tests based on new technology is welcomed. However, it does not avoid the necessity of reviewing correctly stained thick and thin

blood films as the standard operating procedure when malaria is suspected or of replacing a current training program for the identification of the Plasmodium species and for detection of parasitemia below the present threshold of detection by RDT.

CONCLUSION: Falcivax kit showed higher sensitivity and specificity compared to rest of all the rapid methods for detection of P.falciparum.

The logistic, economic and technical factors limit rapid access to microscopic confirmation of malaria in many tropical countries including India. So taking into account of all the factors from our study (Table 6), we consider HRP2 antigen detection kit as a tool for diagnosis of malaria in future. Although no test can replace the conventional method of peripheral blood smear examination, these newer diagnostic tests can be used as supplement to microscopic examination of peripheral blood smear where the diagnosis cannot be made on microscopy alone. These tests can be used in times of urgency and in areas where facilities of microscopy are not available especially during night times when services of laboratory and experienced microscopists are not available.

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TABLE 1 : Sex distribution of clinically suspected patients of malaria (n=339)

	MALES No. (%)	**FEMALES No. (%)**	**TOTAL**
Clinically suspected patients	**238 (70%)**	**101(30%)**	**339**

TABLE 2: Age group Leishman stained thick blood smear positive cases for malaria

			P.falciparum	P.vivax	Mix	Total
Age group	15-25 yrs	Count	11	13	5	29
		% within age group	37.90%	44.80%	17.20%	100.00%
	25-50 yrs	Count	53	31	14	98
		% within age group	53.5%	31.2%	14.3%	100.00%
	50-75 yrs	Count	4	7	0	11
		% within age group	36.40%	63.60%	0.00%	100.00%
Total		Count	68	51	19	138
		% within age group	49.2%	37%	13.8%	100.00%

TABLE 3: Comparison of conventional Leishman stained thick smear examination with other rapid staining methods and rapid immunological techniques for the diagnosis of malarial parasites

	Leishman(gold std)	Leishman stain	JSB stain	Field's stain	Falcivax kit	SD bioline
	Thick Smear	Thin Smear	Thin Smear	Thin Smear		
Negative	201	213	213	213	202	214
PF	68	60	60	60	69	57
PV	51	47	47	47	49	49
MIX	19	19	19	19	19	19
Total	339	339	339	339	339	339

TABLE 4: Comparison of sensitivity and specificity of various rapid methods for P.falciparum with Leishman stained thick smear.

	Leishman	JSB	Field's	Falcivax	SD bioline
	Thin smear	Thin smear	Thin smear		
Sensitivity	88.2 %	88.2%	88.2%	100 %	83.8%
Specificity	100%	100%	100 %	99.6%	100%
PPV	100 %	100%	100%	98.5%	100%
NPV	97%	97%	97%	100%	96.2
test efficiency (Concordance of tests)	97.6%	97.6%	97.6%	99.7	96.7

TABLE 5: Comparison of sensitivity and specificity of various rapid methods for P.vivax with Leishman stained thick smear.

	Leishman	JSB	Field's	Falcivax	SD bioline
	Thin Smear	Thin smear	Thin smear		
Sensitivity	92.0 %	92.0%	92.0%	96.0%	96.0%
Specificity	100%	100%	100%	100%	100%
PPV	100 %	100%	100%	100%	100%
NPV	98.6%	98.6%	98.6%	99.3%	99.3
Test efficiency (Concordance of tests)	98.8%	98.8%	98.8%	99.4	99.4

For mixed malarial parasite infection, results by all the methods were same as compared to the gold standard.

TABLE 6: Comparative evaluation of different methods based on following parameters for diagnosis of malaria.

	Parameters	**Leishman thick smear Stain**	**Leishman thin smear stain**	**JSB stain**	**Field's Stain**	**Falcivax Kit**	**SD Bioline kit**
1	Sensitivity	80parasites/µl	240parasites/µl	240parasites/µl	240parasites/µl	80parasites/µl	200parasites/µl
2	Specificity	All species	All species	All species	All species	P.falciparum and p.vivax	P.falicparum and P.vivax
3	Cost	Rs 5/test	Rs5/test	Rs 5/test	Rs 5 /test	Rs. 100/test	Rs 60/ test
4	Technical expertise	Highly skilled	Highly skilled	highly skilled	Highly skilled	no special skills	minimal skills
5	Equipment	lab, microscope, electricity	Lab, microscope, electricity	lab, microscope, electricity	lab, microscope, electricity	Kit only. no special equipment	Kit only, No special equipment
6	Time for result	45-60mins	30-45mins	20-30 mins	20-30 mins	5 -15 mins	5 -15mins
7	Subjectivity Variation	High	High	High	High	Low	Low

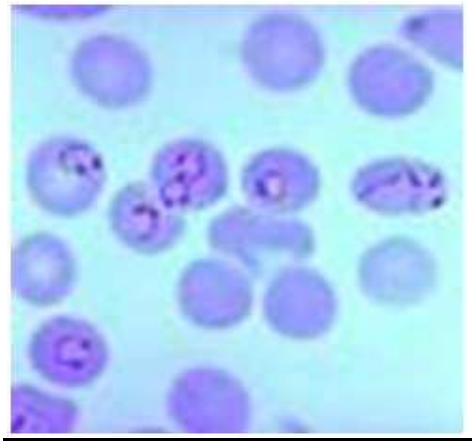


Fig.1 JSB Stained thin smear: P.falciparum rings – multiple rings in one RBC, accole forms

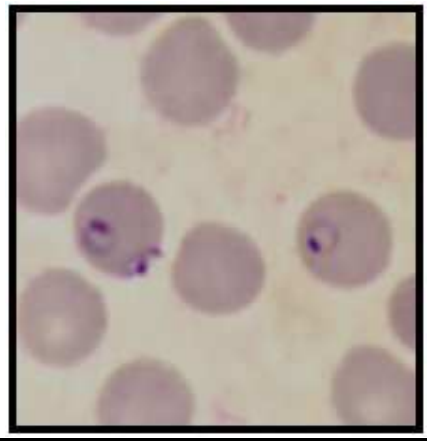


Fig.2 Field's stained thin smear : multiple rings of P.falciparum.

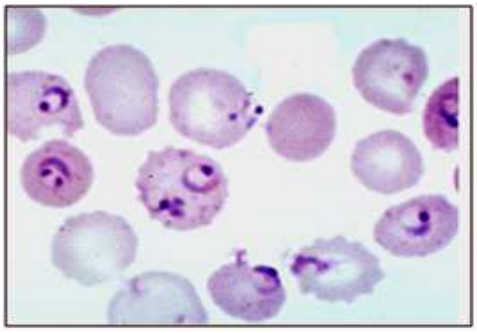


Fig 3. Leishman stained thin smear:: P.falciparum multiple rings, accole forms

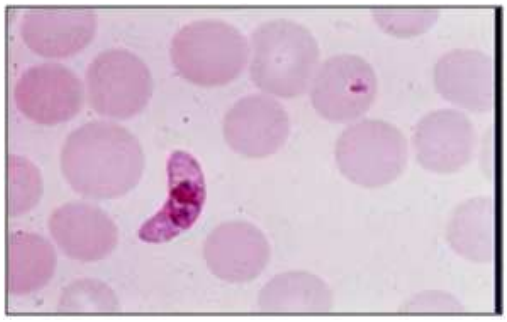


Fig4. JSB stained thin smear: P.falciparum gametocyte

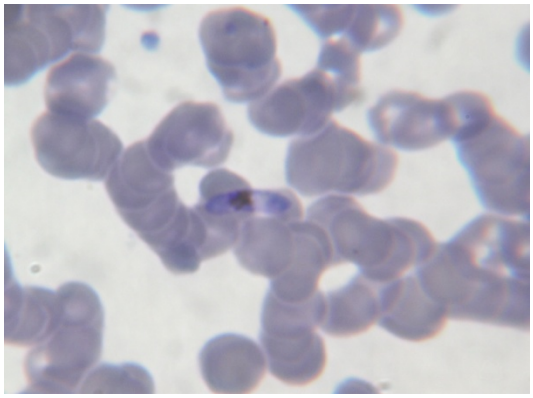


Fig 5. Fields stained thin smear: P.falciparum gametocyte

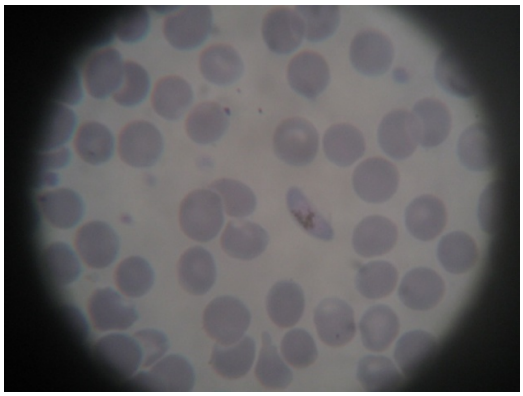


Fig 6. Leishman stained thin smear: P.falciparum gametocyte

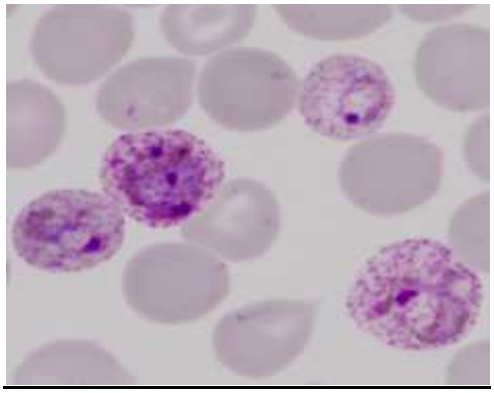


Fig 7. Field's stained thin smear: P. vivax trophozoites (Ring) with Schuffner's dots in RBC cytoplasm & Schizonts of P.vivax

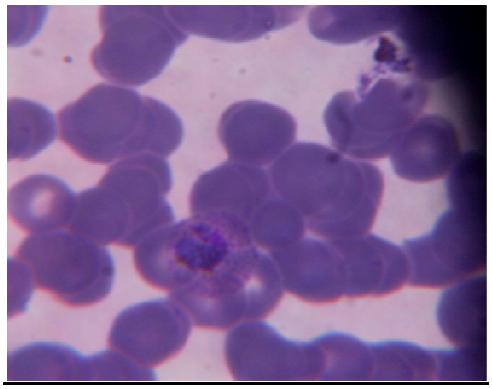


Fig 8. Leishman stained Thin smear: P. vivax schizont

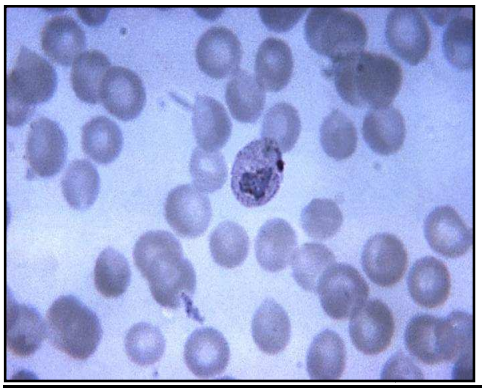


Fig 9. Leishman stain: Schizont of schizont

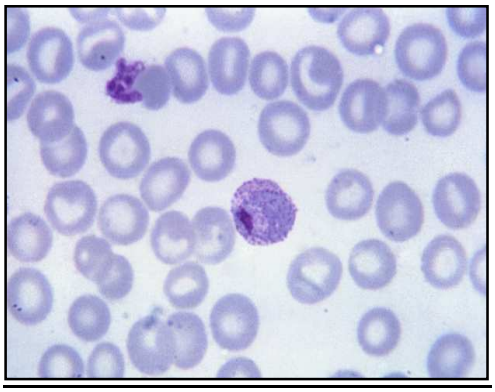


Fig 10. JSB stained thin smear; P.vivax P.vivax

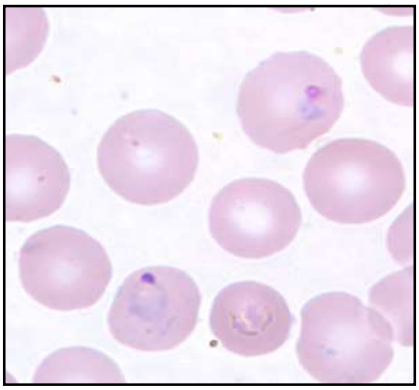


Fig 11. Lesihman's Stain: Ring form of P.vivax

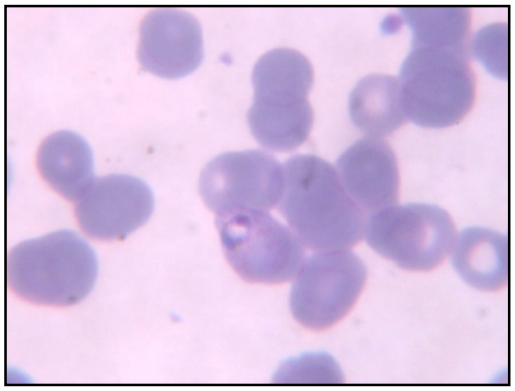


Fig 12. Field's Stain: Ring form of P.vivax

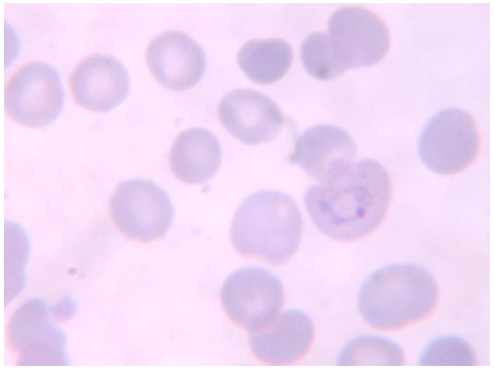


Fig 13. JSB stain : Ring form of P.vivax

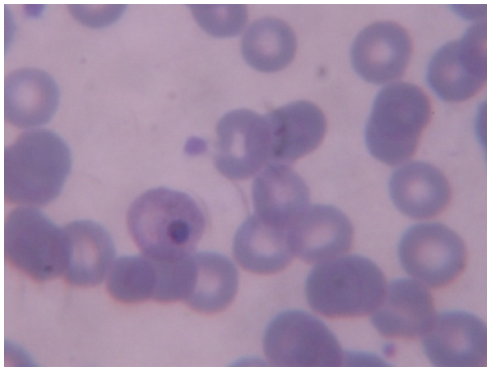


Fig14. JSB Stain: Gametocyte of P.vivax

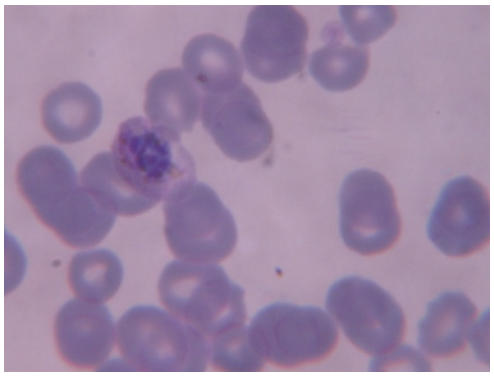
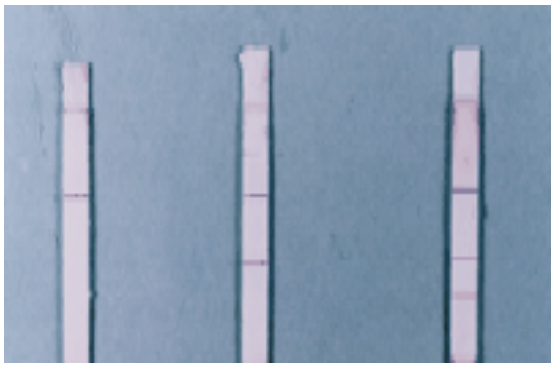


Fig 15. JSB Stain: Schizont with merozoites



1. Negative 2.P. vivax 3. P.falciparum
Fig.16. Interpretation of the test

1-P.falciparum 2- P.vivax C- control

Fig17. Test is positive for P.falciparum and P.vivax

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Research Article Open Access

Evaluation of Blood Smears, Quantitative Buffy Coat and Rapid Diagnostic Tests in the Diagnosis of Malaria

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Abstract

Rapid diagnosis of malaria is important for the administration of effective treatment, to reduce the morbidity and mortality. The present study was carried out to compare the efficacy of quantitative buffy coat (QBC) and rapid diagnostic test (RDT) with conventional peripheral blood smears. Blood samples from 100 patients were obtained with symptoms suggestive of malaria. A total of 74(74%) cases were positive by blood smears, while 80(80%) and 71(71%), were positive by QBC and RDT(Falcivax). Blood smears indicated that 74% (55 0f 74) of the patients were positive for *P.vivax* and 25% (19 of 74) were infected with *P.falciparum*. QBC showed that 75 % (60 0f 80) were positive for *P.vivax* and 25% (20 of 80) were infected with *P.falciparum*. Falcivax identified 74 % (53 of 71) were positive for *P.vivax* and 25 % (18 of 71) of *P.falciparum*. QBC had a sensitivity and specificity of 74.3% and 80.7% for *P.vivax* and 100% and 98.7% for *P.falciparum*. Falcivax had a specificity of100% and sensitivity of 96.3% and 94.7%.

Keywords: Malaria diagnosis; QBC; RDT

Introduction

Malaria, a widely prevalent parasitic disease affects 500 million people each year and is associated with 2-5 million deaths [1]. One of the most pronounced problems in controlling the morbidity and mortality is limited access to effective diagnosis and treatment in areas where malaria is endemic [2]. Microscopic examination of blood smears is the widely used method for detection of malaria parasites and remains the gold standard for malaria diagnosis [3]. But microscopic examination is laborious and time consuming and requires considerable expertise for its interpretation particularly at low levels of parasitemia [4]. Rapid and early detection of malarial parasite and early treatment of infection still remains the most important goals of disease management [5]. A key feature of the World Health Organization global malaria control strategy is the rapid diagnosis of malaria at the village and district level so that effective treatment can be administered quickly to reduce morbidity and mortality. There is therefore an urgent need for a field test which is simple, rapid and accurate. These RDT's have a number of important limitations, including suboptimal sensitivity at low parasite densities, to quantify infection rate and a higher unit cost relative to microscopy [6].

Materials and Methods

This study was conducted in the department of microbiology, Kasturba Medical College Hospital, Ambedkar circle, Mangalore, during the period from July 2005-2007. The study was cleared by the Institutional ethics committee. Patients attending the hospital, with symptoms and signs suggestive of malaria formed the study group. A total of 100 patients were included in the study. Blood sample collected from the patients were subjected to thick and thin smear (Traditional microscopy), Quantitative buffy coat (QBC) and Immuochromatographic test (ICT) Falcivax. Thick and thin smear were stained with Giemsa stain and observed under 100 X microscopy. Thick smear was used for the identification and thin smear for the speciation of the parasite. According to standard practice, thin smear was examined for 15 minutes and thick smear 200 fields were visualized.

Quantitative buffy coat

The QBC capillary tubes were filled with blood by capillary action and were centrifuged at the rate of 1200g for 5 min after proper balancing. The tubes were examined under fluorescence microscope. The ring forms appeared as apple green with or without an orange dot at one side, schizonts as dark brown in colour, and gametocytes as yellowish green sickle shaped bodies.

Immunochromatographic test

Falcivax [Tulip diagnostics pvt ltd, Goa, India], is a rapid self performing, qualitative, immunoassay used for the detection of *P.falciparum* specific histidine rich protein-2 (HRP-2) antigen and *P.vivax* specific lactate dehydrogenase (PLDH).The test was performed according to the manufacturer's instructions, all the kit components were brought to room temperature, the whole blood was centrifuged, and 2-3 drops of serum was dispensed into the sample port, followed by 5 drops of buffer solution provided along with the kit. The results were read at the end of 15 minutes. A pink purple band appeared at the region 'Pv' in the test window 'T' in addition to the control band it was considered as *P.vivax* positive. A pink purple band appeared at the region 'Pf' in the test window 'T' in addition to the control band, it was considered as *P.falciparum* positive.

To measure the agreement between Blood smears, QBC and Falcivax, Kappa statistics was used and statistical significance was assessed.

Results

A total of 100 samples were examined for malaria parasites by quantitative buffy coat and Falcivax and the results were compared with peripheral blood smear examination. Blood smear results indicated that 74 cases were found to be positive for malaria parasites and the rest 24 were negative. Among the positive patients *P.vivax* was detected in 55 cases (75%) and *P.falciparum* in 19 cases (25%).

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Correspondingly QBC method detected, 80(80%) of total malaria cases, of which 60 (75%) cases were positive for *P.vivax* and 20 (25%) cases were positive for *P.falciparum* (Table1). QBC detected five cases of *P.vivax* and one case of *P.falciparum* that were negative by blood smear.

Falcivax indentified 71(71%) of total malaria cases, of which 53(74%) and18 (25%) cases were positive for *P.vivax* and *P.falciparum* infections (Table1). Two cases of *P.vivax* and one case of *P.falciparum* positive by blood smears were not detected by Falcivax.

Sensitivity, specificity, positive and negative predicative value of QBC for *P.vivax* were 91.6,100, 100 and 88.8% respectively and for *P.falciparum* were 95,100, 100 and 8.7% where that of Falcivax were 100, 95.7, 96.3 and 100% for *P.vivax* and 100, 98.7, 94.7 and 100% for *P.falciparum* (Table 2).

On comparing, QBC test with blood smear examination for *P.vivax* (K=0.898, P<0.0001) and for *P.falciparum* (K=0.968, P <0.0001) which is statistically significant. Comparison of Falcivax with peripheral blood smear examination for *P.vivax* (K=0.960, P< 0.0001) and for *P.falciparum* (K=--, P <0.0001) which is also statistically significant.

Discussion

Malaria is a well-known disease and it continues to be a major public health problem at the start of new millennium. Reliable diagnosis of malaria requires laboratory confirmation of the presence of malaria parasites in the blood of a febrile patient [7]. Although microscopic examination of blood smear continues to be the gold standard, it has a drawback that it is time consuming and requires an expert microscopist and less sensitive in cases of low parasitemia [8]. Various sensitive methods have been employed for the simple, reliable, and rapid diagnosis of malaria, the most promising of these is the rapid diagnostic test and quantitative buffy coat [9]. We employed these tests and compared with Giemsa stained peripheral blood smear for the diagnosis of *P.vivax* and *P.falciparum* infections.

The QBC and RDT identified 80% and 71% as malaria positive while blood smears detected 74% of the positive cases. Five cases of *P.vivax* and one case of *P.falciparum* negative by blood smear were detected by QBC indicating a higher sensitivity and specificity of QBC. High sensitivity of QBC might be due to concentration of parasites below the buffy coat. Parzy et al found QBC to be more sensitive than blood smear examination and advocated its use for urgent diagnosis [10]. In our study the sensitivity and specificity of QBC for *P.vivax* was74.3% and 80.7% and for *P.falciparum* was100% and 98.67% respectively our results are in agreement with the results reported by various studies. Study by (Ye Htut et al. 2002) had a sensitivity of 82.8% and 100% for *P.falciparum* and *P.vivax* and specificity of 97.1% and 98.6% [11].

One of the major advantages of the QBC technique is rapidity and reliability in diagnosis of malaria even under field conditions. In addition, it requires less training and experience than blood smears. Its chief drawback is its high cost and in the identification of *Plasmodium* species. Ring stages of *P. falciparum* and *P. vivax* are difficult to distinguish by the QBC. This problem is particularly important in endemic areas where *P.falciparum* coexists with *P. vivax* [12].

Blood smear	QBC		Falcivax	
P.vivax	+	-	+	-
Positive-55	55	0	53	2
Negative-45	5	40	0	45
Total-100	60	40	53	47
P.falciparum				
Positive-19	19	0	18	1
Negative-81	1	80	0	81
Total-100	20	80	18	82

Table 1: Comparison of peripheral blood smears with other methods for the detection of malaria parasites

	QBC		Falcivax	
	P.vivax	***P.falciparum***	***P.vivax***	***P.falciparum***
Sensitivity (%)	91.6	95	100	100
Specificity (%)	100	100	95.7	98.7
Positive predictive value (%)	100	100	96.3	94.7
Negative predictive value (%)	88.8	98.7	100	100

Table 2: Comparison of sensitivity and specificity of various methods in the identification of malarial parasites

Falcivax failed to detect two cases of *P.vivax* and one case of *P.falciparum* which were positive by blood smears. The sensitivity and specificity of falcivax was 96.3% and 100% for *P.vivax* and 94.7% and100% for *P.falciparum*. The low sensitivity of the Falcivax can be explained by the fact that it detects enzyme pLDH produced by live parasites and the parasites might have been killed and not cleared from the host[4] and also due to low parasitemic levels as observed by Iqbal et al. who observed 75% sensitivity at parasitemia < 100/µl. However, the rapid diagnostic test was found to be user friendly and interpretation was more objective as compared to smear and QBC [13]. Although no single test can replace the conventional method of peripheral blood smear examination, these newer diagnostic tests can be used as supplement to microscopic examination of peripheral blood smear where the diagnosis cannot be made on microscopy and an experienced microscopists are not available. The high cost of the test may prevent routine use in many laboratories. However it is a valuable adjuvant at the time of emergency for rapid diagnosis, although microscopy remains the main stay for the diagnosis of malaria.

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Real-time PCR assay and rapid diagnostic tests for the diagnosis of clinically suspected malaria patients in Bangladesh

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Abstract

Background: More than 95% of total malaria cases in Bangladesh are reported from the 13 high endemic districts. *Plasmodium falciparum* and *Plasmodium vivax* are the two most abundant malaria parasites in the country. To improve the detection and management of malaria patients, the National Malaria Control Programme (NMCP) has been using rapid diagnostic test (RDT) in the endemic areas. A study was conducted to establish a SYBR Green-based modified real-time PCR assay as a gold standard to evaluate the performance of four commercially-available malaria RDTs, along with the classical gold standard- microscopy.

Methods: Blood samples were collected from 338 febrile patients referred for the diagnosis of malaria by the attending physician at Matiranga
Upazila Health Complex (UHC) from May 2009 to August 2010. Paracheck RDT and microscopy were performed at the UHC. The blood samples were preserved in EDTA tubes. A SYBR Green-based real-time PCR assay was performed and evaluated. The performances of the remaining three RDTs (Falcivax, Onsite Pf and Onsite Pf/Pv) were also evaluated against microscopy and real-time PCR using the stored blood samples.

Result: In total, 338 febrile patients were enrolled in the study. Malaria parasites were detected in 189 (55.9%) and 188 (55.6%) patients by microscopy and real-time PCR respectively. Among the RDTs, the highest sensitivity for the detection of *P. falciparum* (including mixed infection) was obtained by Paracheck [98.8%, 95% confidence interval (CI) 95.8-99.9] and Falcivax (97.6%, 95% CI 94.1-99.4) compared to microscopy and real-time PCR respectively. Paracheck and Onsite Pf/Pv gave the highest specificity (98.8%, 95% CI 95.7-99.9) compared to microscopy and Onsite Pf/Pv (98.8, 95% CI 95.8-99.9) compared to real-time PCR respectively for the detection of *P. falciparum*. On the other hand Falcivax and Onsite Pf/Pv had equal sensitivity (90.5%, 95% CI 69.6-98.8) and almost 100% specificity compared to microscopy for the detection of *P. vivax*. However, compared to real-time PCR assay RDTs and microscopy gave low sensitivity (76.9%, 95% CI 56.4-91) in detecting of *P. vivax* although a very high specificity was obtained (99- 100%).

Conclusion: The results of this study suggest that the SYBR Green-based real-time PCR assay could be used as an alternative gold standard method in a reference setting. Commercially-available RDTs used in the study are quite sensitive and specific in detecting *P. falciparum*, although their sensitivity in detecting *P. vivax* was not satisfactory compared to the real-time PCR assay.

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Background

Malaria is still considered a major public-health problem in the eastern districts of Bangladesh, bordering India and Myanmar. These districts experience a perennial transmission of malaria with two peaks in pre-monsoon (March-May) and post-monsoon (September-November) periods [1]. In the changing climatic situation and in absence of major malaria vectors, such as *Anopheles minimus* and *Anopheles baimaii* a number of *Anopheles* species have been incriminated and playing a role in the transmission of malaria in the country [2]. *Plasmodium falciparum* and *Plasmodium vivax* are two main malaria parasites in the country as reported by a nationwide prevalence survey in 2007. The survey showed that contribution of *P. falciparum* was 90.18%, followed by *P. vivax* (5.29%), and the remaining (4.53%) was mixed infection of these two species [3].

The Giemsa-stained blood slide using thin and thick smears for malaria parasites has been the gold standard method for nearly a century [4]. No alternative method still could be established to replace this universally-accepted gold standard method. Such a laboratory technique to confirm the clinical suspicion of malaria is labour-intensive [5] and sometimes unreliable due to lack of skilled microscopists, limited supplies, inadequate maintenance of microscopes and reagents, and inadequate or absence of quality-control systems [6].

In recent time, lateral flow immunochromatographic-based rapid diagnostic test (RDT) has been developed for the diagnosis of suspected malaria patients and are widely used in remote areas across the world [7]. Most RDTs are intended to react with antigens commonly released from or enzymes present in parasitized red blood cells. In the case of *P. falciparum*, the water soluble histidine-rich protein-2 (HRP-2) antigen is commonly used as it is specific to *P. falciparum* associated infection. Non-falciparum malaria or mixed infections with *P. falciparum* are commonly detected by *Plasmodium* lactate dehydrogenase (pLDH) [8,9]. In the Global Fund sponsored malaria control programme RDT is recommended and being widely used for detecting malaria cases in the endemic areas of Bangladesh [1].

The molecular detection method, such as polymerase chain reaction (PCR) has been developed to diagnose *Plasmodium* spp. and has been performed in several places for routine diagnosis or for evaluating the performance of microscopy or RDT [10-14]. In recent time, real-time PCR method has been established for the quantitative detection of malaria parasites [15-19]. Real-time PCR is reliable and yield high sensitivity and specificity when compared with microcopy or nested PCR [15,19,20].

This study demonstrated a SYBR Green-based modified real-time method to use it as a gold standard, along with conventional microscopy to evaluate four RDTs for diagnosis of malaria from suspected febrile patients. Such a study has never been done before in Bangladesh. The study would provide additional support to the NMCP for monitoring and evaluation of the performances of the diagnostic methods used in their ongoing malaria control programme.

Methods

Study area and population

The study was conducted at Matiranga Upazila (sub-district) of Khagrachari district situated at the south-eastern part of Bangladesh. Febrile patients referred to microscopy for malaria diagnosis at Matiranga Upazila Health Complex (UHC) from May 2009 to August 2010 were enrolled. The recent malaria prevalence survey, Matiranga showed high prevalence of asymptomatic malaria cases (21.6%) [21].

Sample collection

Five ml of blood was taken from an adult subject and in case of children or minor subjects three ml of blood was obtained through venipuncture by an experienced medical technologist. Two drops of sample were used for preparing thick and thin smear slides, one drop was used for Paracheck RDT, and the remaining samples were preserved in an EDTA tube and stored at -20°C.

Microscopy

The blood film was stained with Giemsa in phosphate buffer saline and examined under the compound microscope at a magnification of ×1,000 for malaria parasites. Blood films were defined as negative if no parasite was observed in 100× oil immersion fields (magnification, ×1,000) on thin film by an experienced microscopist [22]. Declaring a slide positive or negative and initial speciation was routinely based on the examination of 200 fields in the Giemsa-stained thick film. A slide was considered positive when at least one parasite was found. After finding the first parasite, another 200 fields were completed for any mixed infection. If no parasite was found in 200 oil fields, the slide was considered negative. Density of the parasite was measured from thick blood smears by counting the number of parasites per 200 leukocytes and expressed as parasites/μl. In the case of 10 or less parasites, 500 leukocytes were counted. Each slide was assessed by two independent microscopists; one of them was employed by the study and the other person was posted at Matiranga UHC. A slide was considered positive only when these two microscopists were in agreement. There was a provision for third microscopist posted at the Khagrachari Civil Surgeon's office situated 20 km away from Matiranga UHC for any disagreement between them.

Rapid diagnostic tests

In the present investigation four RDTs (device) were used. These were Paracheck (Orchid Biomedical System, India), FalciVax Pf (Zephyr Biomedicals, India), Onsite Pf (CTK Biotech Inc, USA) and Onsite Pf/Pv (CTK Biotech Inc, USA). Paracheck and Onsite Pf used *P. falciparum*-specific HRP-2 antigen. FalciVax and Onsite Pf/Pv used *P. vivax*- specific pLDH together with *P. falciparum*-specific HRP-2.

All the RDTs were used following the instructions of the manufacturers. 'Paracheck' is being used by the National Malaria Control Programme (NMCP) in the endemic areas and was available at the MUHC. Paracheck test was performed at Matiranga UHC concurrently with the microscopy. The remaining three RDTs were performed using the stored samples as per the instructions of the manufacturers.

DNA extraction

DNA was extracted from 200 μl EDTA preserved blood samples using the QiaAmp blood mini kit (QIAGEN, Inc., Germany) following the manufacturer's instructions at the Parasitology Laboratory of ICDDR,B. DNA sample was stored at 4°C until PCR could be completed.

Real-time PCR

Real-time PCR was done by the primer sets described by Perandin *et al* [15] with some modification to a singleplex reaction. Instead of TaqMan probe, SYBR Green I dye was used for visualizing the amplification. PCR condition was also modified slightly to fit with Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen Corporation, USA) following the instructions of the manufacturer. Purified DNA templates were amplified in a BioRad CFX-96 real time system (BioRad, USA) with a species-specific primer set. Briefly, a 25-μl PCR mixture was prepared using 1 μl of template DNA, 12.5 μl Platinum SYBR Green qPCR supermix (PlatinumR *Taq* DNA polymerase, SYBR Green I dye, Tris-HCl, KCl, 6 mM MgCl2, 400 μM dGTP, 400 μM dATP, 400 μM dCTP, 800 μM dUTP, uracil DNA glycosylase, and stabilizers), 320 nM concentration of each of parasite species-specific primer set. Amplification and detection were performed as follows: 50°C for 2 min and 95°C for 2 min. After that 95°C for 1 min, 58°C for 1 min and 72°C for 1 min 30 sec for a single cycle were performed. 40 cycles were considered for *P. falciparum* and 35 cycles for *P. vivax*. The plate read was taken after the extension at 72°C. The melt curve was prepared from 50°C to 95°C with an increment of 0.5°C each after five seconds.

To establish the minimum number of parasites detectable by the *Plasmodium* SYBR Green assay (detection limit), blood samples from two patients infected, respectively, with *P. falciparum* (one patient) and *P. vivax* (one patient) were collected, and parasitaemia was calculated using 200 WBC count as reference. The infected blood samples were diluted with uninfected erythrocytes from healthy individuals with known baseline erythrocyte counts. Ten-fold serial dilution was made to obtain a final parasitaemia of 1% (1 parasite/μl of blood) for each sample. All DNA aliquots purified from the dilutions were treated in duplicate for real-time PCR assay. To estimate the analytical specificity of the *Plasmodium* real-time PCR assay, DNA from *in vitro* culture samples of other protozoan parasites, such as *Entamoeba histolytica* and *Leishmania donovani* were used. The clinical sensitivity and specificity of the modified *Plasmodium* real-time PCR assay for detecting and identifying malaria parasites were calculated on 338 whole-blood samples, microscopy as the gold standard and vice- versa.

Analysis of data

The performance of each method was calculated by means of sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) using microscopy and modified real-time PCR as gold standard. SPSS software version 11.5 (SPSS Inc., USA) was used for calculating the kappa coefficient (k) of the tests for each association using the X^2 test. Sensitivity, specificity, positive predictive value and negative predictive value were calculated using the 'diagt' command of the STATA software version 10 (Stata Corp, USA)[23].

Ethical approval

The study was approved by the Research Review Committee and Ethical Review Committee of ICDDR,B. Approval was also obtained from the NMCP for the study. Informed consent was obtained from all adult subjects, and assent was obtained from the legal guardians in the case of minor subjects before the collection of blood sample. Good clinical and laboratory practices were followed in all the procedures.

Results

Enrollment

In total, 338 febrile patients were recruited for the study from May 2009 to August 2010. Of them, 50.3% were female. The age of the patients ranged from 18 months to 82 years, with a median age of 14 years.

Microscopy

Malaria parasites were detected in 189 (55.9%) patients by microscopy. Of them 168 (88.9%) were infected by *P. falciparum*, 18 (9.5%) patients by *P. vivax* and remaining three (1.6%) patients had a mixed infection of *P. falciparum* and *P. vivax* (Table 1). Overall, high parasite

Table 1 Results for different tests used in the study

Test	Negative	Positive			
	N (%)	Pf (%)	Pv (%)	Pf + Pv mixed (%)	Total (%)
Microscopy	149 (44.1)	168 (49.7)	18 (5.3)	3 (0.9)	189(55.9)
Paracheck	167 (49.4)	171 (50.6)	N/A	N/A	171 (50.6)
Onsite Pf	174 (51.5)	164(48.5)	N/A	N/A	164(48.5)
Falcivax	147 (43.5)	171 (50.6)	18 (5.3)	2 (0.6)	191 (56.5)
Onsite Pf/Pv	160 (47.3)	155 (45.9)	18 (5.3)	5 (1.5)	178 (52.7)
Real-time PCR	150 (44.4)	162 (47.9)	18 (5.3)	8 (2.4)	188(55.6)

count was observed in microscopy. Parasite count ranged from 16 to 261,480 parasites/μl of blood. A median number of parasite count of 19,960 [interquartile range (IQR) 6,280-48,320] parasites/μl of blood was found in 171 *P. falciparum* positive patients. Only six (3.5%) of the samples were below 100 parasites/ μl whereas 118 (69%) had a count of more than 10,000 parasites/ μl of blood. Of 21 *P. vivax* positive slides, the parasite count ranged from 32 to 25,120 parasites/μl of blood, with a median of 5,040 (IQR 520-17,160) parasites/μl of blood. One (4.8%) sample had a parasite count below 100 parasites/ μl whereas 15 (71.4%) of the sample had a count of more than 1,000 parasites/μl of blood (see Additional file 1).

Real-time PCR

Typical displays (amplification plots) for *P. falciparum* and *P. vivax* by the SYBR Green I PCR assay provided by Bio Rad CFX-96 are shown in Figures 1 and 2. Positive signals by means of cycle threshold [CT] value were obtained for all dilutions, with a detection limit of 5-10 parasites/μl for *P. falciparum* and *P. vivax* in different experiments. Reproducible linearity of over a 10,000-fold range was shown by CT values. A significant correlation coefficient was found for the mean CT values and parasitaemia (*P. falciparum*, R^2 = 0.982; *P. vivax*, R^2 = 0.994) (Figures 3 and 4). For non-*Plasmodium* protozoan DNA (*E. histolytica* and *L. donovani*) and blood DNA samples of healthy human subjects no signal was obtained by the SYBR Green real-time PCR. The melt peak for *P. falciparum and P. vivax* was found at 74.5°C and 75.5°C from the corresponding positive controls respectively (Figures 5 and 6). Any amplification other than these two melting temperatures was excluded as false amplification.

Using the real-time PCR assay results 188 (55.6%) samples were found positive for any malarial infection (Table 1). Of the 188 PCR positive samples 162 (86.2%) were infected by *P. falciparum*, 18 (9.5%) were infected by *P. vivax* and the remaining 8 (4.3%) samples were mixed infection with *P. falciparum and P. vivax* (Table 1). Sensitivity, specificity, positive predictive value, negative predictive value, and kappa (k) of PCR assay compared to microscopy are given in table 2. For the detection of *P. falciparum* (including mixed infection), modified real-time PCR assay had 97.1% (95% CI: 93.3-99) sensitivity and 97.6% (95% CI: 94-99.3) specificity respectively. While for the detection of *P. vivax* (including mixed infection) modified real-time PCR showed 95.2% (95% CI: 76.2-99.9) sensitivity and 98.1% (95% CI: 95.9-99.6) specificity respectively (Table 2).

Compared to real-time PCR assay, microscopy had 97.6% sensitivity (95% CI: 94.1-99.4) and 97% (95%CI

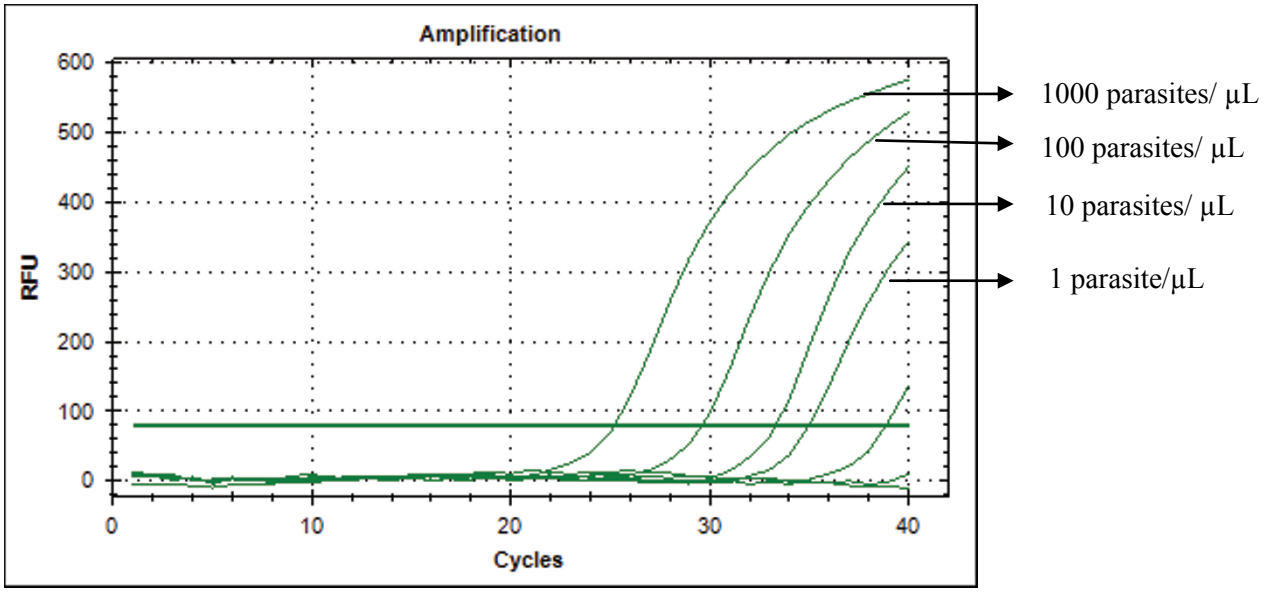

Figure 1 Typical amplification curve (generated by CFX-96 Real-Time System) for *P. falciparum*.

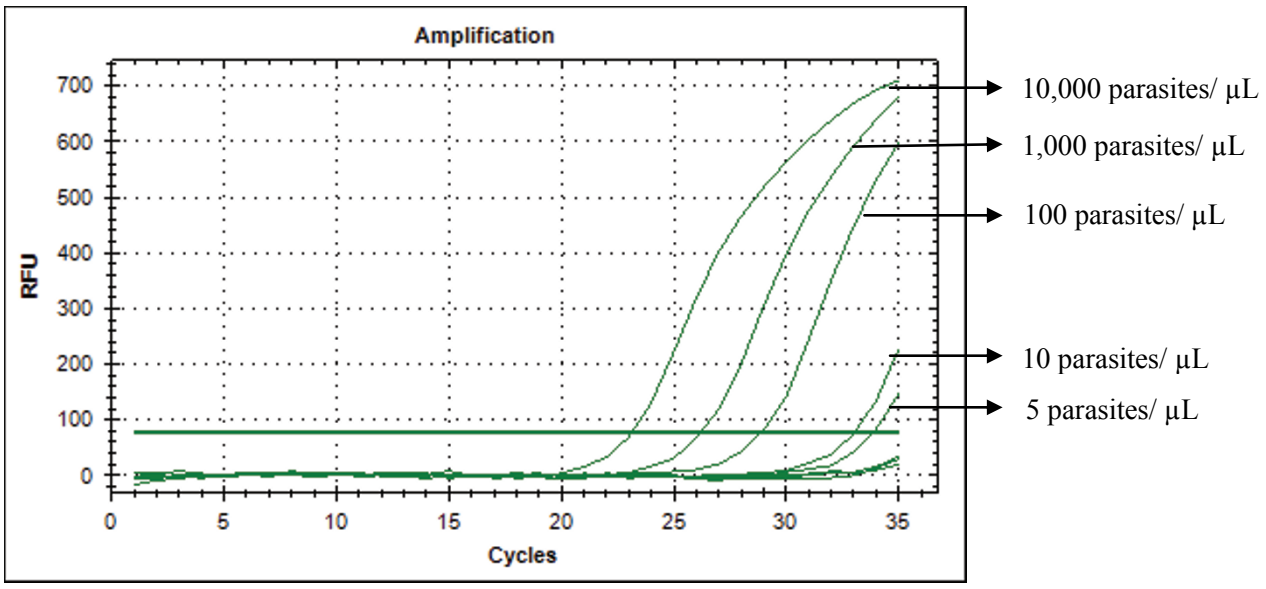

Figure 2 Typical amplification curve (generated by CFX-96 Real-Time System) for *P. vivax*.

93.2-99) specificity for the detection of *P. falciparum* and 76.9% sensitivity (95% CI: 56.4-91) and 99.7% specificity (95% CI: 98.2-100) for the detection of *P. vivax* respectively (Table 3).

Rapid diagnostic tests

Of the four RDTs used in this study, Onsite Pf and Paracheck can detect *P. falciparum* only. Sensitivity, specificity, positive predictive value, and negative predictive value for each of the RDTs compared to microscopy (gold standard) and real-time PCR, are given in Table 2 and 3 respectively. Of the RDTs, the highest sensitivities for detection of *P. falciparum* (including mixed infection) were obtained by the Paracheck (98.8%, 95% CI 95.8-99.9) compared to microscopy and Falcivax (97.6%, 95% CI 94.1-99.4) compared to real-time PCR assay respectively. Although Paracheck and Onsite Pf/Pv gave the highest specificity 98.8% (95% CI 95.7-99.9) compared to microscopy and Onsite Pf/Pv 98.8% (95% CI 95.8-99.9) compared to real-time PCR assay respectively. Although both Falcivax and Onsite Pf/Pv had the highest sensitivity (90.5%, 95% CI 69.6-98.8), but Falcivax

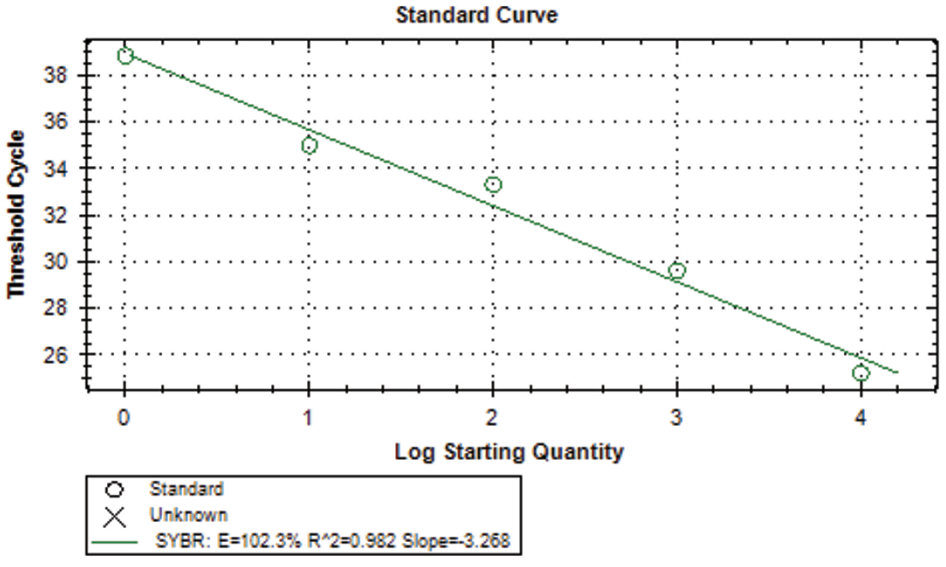

Figure 3 Standard curve for *P. falciparum* produced against CT values and logarithm of parasite count/µL of blood (R^2 = 0.982).

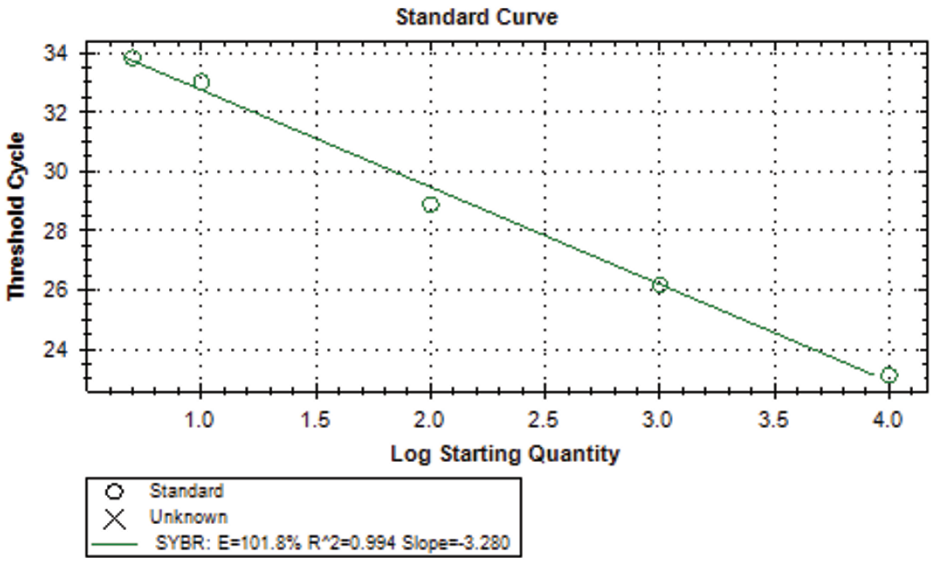

Figure 4 Standard curve for *P. vivax* produced against CT values and logarithm of parasite count/μL of blood (R^2 = 0.994).

had the highest specificity (99.7%, 95%CI 98.3-100) compared to microscopy for the detection of *P. vivax*. However, compared to real-time PCR assay, these RDTs gave low sensitivity (76.9%, 95% CI 56.4-91) although very high specificity (100%, 95%CI: 98.8-100) was obtained for 'Falcivax' and for 'Onsite Pf/Pv' (99%, 95% CI: 97.2-99.8) respectively.

Discussion

Although in Bangladesh, *P. falciparum* and *P. vivax* are the two common prevalent parasites, the majority of malaria cases are caused by *P. falciparum* [1]. However, their ratio varies from time to time. During the nationwide malaria prevalence survey in 2007 based on Falcivax RDT, 90% *P. falciparum* infection was found and the remaining 10% infection was due to pure *P. vivax* or mixed infection of these two parasites although the performance of that RDT (Falcivax) was not evaluated against any gold standard method [3].

This study demonstrated the establishment of pre existing real-time PCR assay modified with SYBR Green dye for the detection of *P. falciparum* and *P. vivax* as an alternative gold standard method for evaluating RDTs used for the diagnosis of malaria. Simultaneously, the

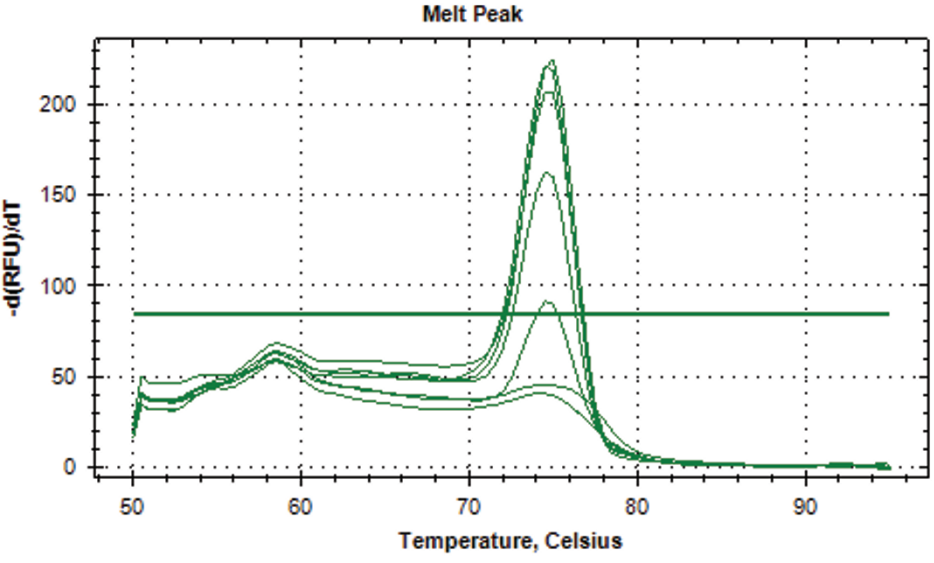

Figure 5 Typical melt curve of *P. falciparum* showing peak at 74.5°C.

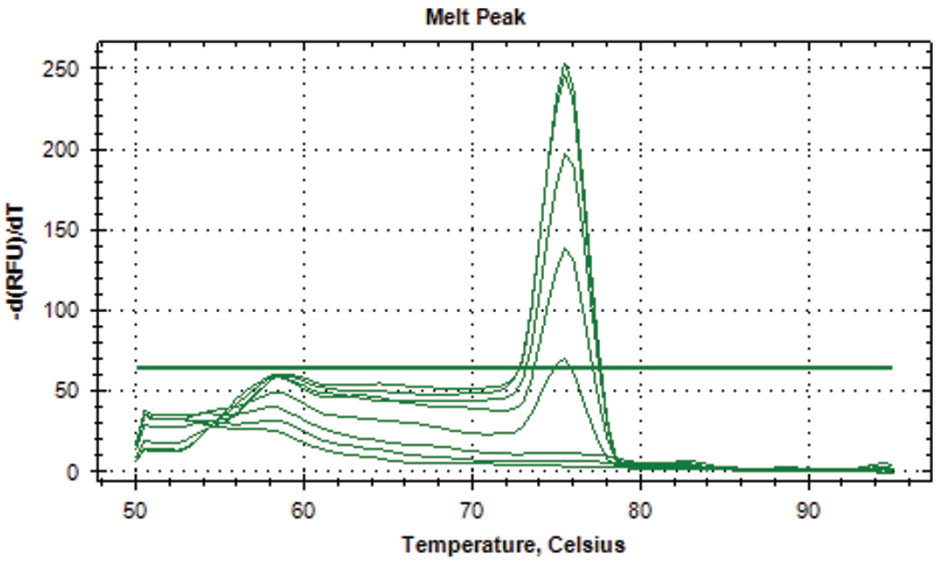

Figure 6 Typical melt curve of *P. vivax* showing peak at 75.5°C.

performance of microscopy can also be evaluated by this SYBR Green-based PCR method. The original TaqMan-based real-time PCR method was 100% sensitive and specific using nested PCR as gold standard [15]. But in the present study more than 95% of sensitivity and specificity was obtained for both *P. falciparum* and *P. vivax* using microscopy as a gold standard. The modified real-time PCR method detected eight *P. falciparum* and *P. vivax* mixed infections, of which two were detected by microscopy, Falcivax and Onsite Pf/Pv RDT. On the other hand microscopy detected three mixed infections of which modified real-time PCR could detect two and one by Falcivax and Onsite Pf/Pv tests respectively. One sample detected as *P. vivax* by microscopy was detected as mixed infection by modified real-time PCR, Falcivax and Onsite Pf/Pv tests. At the same time one sample was found to be *P. vivax* in all other tests, but detected as *P. falciparum* by microscopy. Two samples were detected negative by microscopy and all the RDTs, but found to have *P. falciparum* in real-time PCR. These two samples were missed in microscopy and RDTs perhaps due to the low number of parasite counts [15,24].

This study was conducted among the symptomatic febrile patients in a high-endemic area. RDTs can play a key role in rapid diagnosis and, hence, prompt treatment of malaria. As RDT can be conducted immediately in the field clinic or even in the field level by the health workers while the patient is present, the most important point for the villagers is the knowledge that they are infected with malaria parasite. On the contrary, the delay in the results of microscopic diagnosis is a serious obstacle for the operation of a malaria control programme in remote areas. Although RDTs have some limitations, all the four tests evaluated had high sensitivity and specificity. The high NPV allow us to confidently diagnose negative test patients as non-malaria patients [25]. Thus, the risk of missing an infected individual is less by the RDTs used in this evaluation. In a similar

Table 2 Sensitivity, specificity, positive predictive value, and negative predictive value of RDTs and real-time PCR versus microscopy as gold standard

Method	Test	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95%CI)	Kappa (k)
Paracheck	Pure and mixed Pf	98.8 (95.8-99.9)	98.8 (95.7-99.9)	98.8 (95.8-99.9)	98.8 (95.7-99.8)	0.98
Falcivax	Pure and mixed Pf	98.2 (95-99.6)	97 (93.2-99)	97.1 (93.4-99.1)	98.2 (94.8-99.6)	0.95
Onsite Pf	Pure and mixed Pf	93.6 (88.8-96.7)	97.6 (94-99.3)	97.6 (93.9-99.3)	93.7 (89-96.8)	0.91
Onsite Pf/Pv	Pure and mixed Pf	92.4 (87.4-95.9)	98.8 (95.7-99.9)	98.8 (95.6-99.8)	92.7 (87.8-96.1)	0.91
PCR	Pure and mixed Pf	97.1 (93.3-99)	97.6 (94-99.3)	97.6 (94.1-99.4)	97 (93.2-99)	0.95
Falcivax	Pure and mixed Pv	90.5 (69.6-98.8)	99.7 (98.3-100)	95 (75.1-99.9)	99.4 (97.7-99.9)	0.92
Onsite Pf/Pv	Pure and mixed Pv	90.5 (69.6-98.8)	98.7 (96.8-99.7)	82.6 (61.2-95)	99.4 (97.7-99.9)	0.85
PCR	Pure and mixed Pv	95.2 (76.2-99.9)	98.1 (95.9-99.6)	76.9 (56.4-91)	99.7 (98.2-100)	0.84

(PPV = Positive predictive value, NPV = Negative predictive value).

Table 3 Sensitivity, specificity, positive predictive value and negative predictive value of RDTs and microscopy versus real-time PCR as gold standard

Method	Test	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95%CI)	Kappa (k)
Paracheck	Pure and mixed Pf	97.1 (93.3-99)	96.4 (92.4-98.7)	96.5 (92.5-98.7)	97 (93.2-99)	0.94
Falcivax	Pure and mixed Pf	97.6 (94.1-99.4)	95.8 (91.6-98.3)	96 (91.8-98.4)	97.6 (93.9-99.3)	0.94
Onsite Pf	Pure and mixed Pf	94.1 (89.4-97.1)	97.6 (94-99.3)	97.6 (93.9-99.3)	94.3 (89.7-97.2)	0.92
Onsite Pf/Pv	Pure and mixed Pf	92.9 (88-96.3)	98.8 (95.8-99.9)	98.8 (95.6-99.8)	93.3 (88.5-96.5)	0.92
Microscopy	Pure and mixed Pf	97.6 (94.1-99.4)	97 (93.2-99)	97.1 (93.3-99)	97.6 (94-99.3)	0.95
Falcivax	Pure and mixed Pv	76.9 (56.4-91)	100 (98.8-100)	100 (83.2-100)	98.1 (95.9-99.3)	0.86
Onsite Pf/Pv	Pure and mixed Pv	76.9 (56.4-91)	99 (97.2-99.8)	87 (66.4-97.2)	98.1 (95.9-99.3)	0.80
Microscopy	Pure and mixed Pv	76.9 (56.4-91)	99.7 (98.2-100)	95.2 (76.2-99.9)	98.1 (95.8-99.3)	0.84

(PPV = Positive predictive value, NPV = Negative predictive value).

study in India, high NPV was also recorded for Falcivax RDT [13].

Overall, 55.9% of the febrile patients with suspected malaria in the present study had a positive blood slide, indicating that over half of the suspected cases referred to this hospital (Matiranga UHC) had malaria. A high percentage of malaria cases among the febrile cases of this area could be due to a high prevalence of asymptomatic malaria cases at the community [21].

Pf-HRP 2 based Paracheck is currently being used in the country's NMCP, although there is a necessity of a RDT for detecting multiple malaria infections in the country [1]. In the present study Parachek showed high sensitivity and specificity compared to a study in Malawi where low specificity was reported [26]. However, since the control programme is now targeting for RDTs that can detect multiple infections, this study could provide a valuable guideline to them.

Onsite duo as newly developed test had never been evaluated earlier in any part of the world gave satisfactory results in the present study. Although Onsite Pf/Pv failed to diagnose some *P. falciparum* positive sample which undoubtedly affects its sensitivity compared to its counterpart Falcivax. However, Onsite Pf/Pv gave almost a similar result as like as Falcivax for detecting *P. vivax*.

Low sensitivity of the two RDTs (Falcivax and Onsite Pf/Pv) for the detection of *P. vivax* compared to real-time PCR assay in this present study is similar to studies published earlier [20,26]. This could be due to the inherent limitations of pLDH assay to detect low parasitaemia in the clinical specimens [27]. Miscroscopists similar to RDTs also missed *P. vivax* cases compared to real-time PCR assay perhaps due to the same reason (low parasitaemia)[28].

RDTs do not depend on the operator like microscopy. It was evolved to overcome or reduce the limitations of microscopy. They have brought a revolution in the field of malaria diagnosis. However, these must achieve > 95% sensitivity to prove their usefulness [29]. It has been estimated that over 70 million RDTs are sold across the world. There are a number of companies producing RDTs for the diagnosis of malaria which was initiated by a single company in 1993 [30]. The world health organization listed approximately 50 RDTs, of which only a few had PvLDH antigen-based tests that distinguish between *P. falciparum* and *P. vivax* are commercially available [26].

Conclusions

Findings of the study suggest that the SYBR Green-based real-time PCR and RDTs used in the study are sensitive and specific for the detection of *P. falciparum*. However, RDTs and microscopy were not sensitive enough compared to real-time PCR assay for the detection of *P. vivax*. The SYBR Green-based real-time PCR could be a useful tool for monitoring the performance of different malaria diagnostic tests in a reference setting by the NMCP. Efforts should be given to increase the accuracy of RDTs as well as microscopy for diagnosis of *P. vivax* in the field level. As more than one malaria parasites are present in the endemic areas of Bangladesh, it is imperative to deploy a RDT that can detect multiple malaria infections by the NMCP.

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Authors' contributions
MSA conceptualized and designed the study, collected and identified samples, analysed data, drafted the manuscript and made final revisions. MSA, ANM, WAK, NI, MJK, HK, DS, RH did sample analysis and made critical revision of the manuscript. SM organized the field activities, analysed data and helped revise the manuscript. MSA and RH drafted the manuscript. All the authors read the final version of the manuscript and approved.

Competing interests
The authors declare that they have no competing interests.

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Accuracy of a multispecies rapid diagnostic test kit for detection of malarial parasite at the point of care in a low endemicity region

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Summary Although highly accurate rapid diagnostic tests (RDT) for *Plasmodium falciparum* [based on identification of histidine-rich protein-2 (PfHRP2)] have been developed, the accuracy of non-falciparum tests is relatively poor. Recently, a *Plasmodium vivax*-specific RDT [based on identification of species-specific lactate dehydrogenase (PvLDH)] became available, which along with PfHRP2 may improve malaria diagnosis by identifying the species correctly. A cross-sectional hospital-based study was designed to evaluate the diagnostic accuracy of FalciVax, a commercially available PfHRP2- and PvLDH-based RDT (index test), using malaria microscopy as a reference standard. All consecutive inpatients who presented with fever underwent both the index test and the reference standard. The study sample included 657 patients and the overall sensitivity and specificity of the RDT for diagnosis of any malarial species were 92.9% and 98.4%, respectively. The diagnostic accuracy estimates for correct species identification were lower (sensitivity 91.8%, specificity 96.8%). The accuracy of the PvLDH test to detect *P. vivax* was low (sensitivity 76.6%, specificity 98.1%).

1. Introduction

Malaria is an important public health problem in India, with 1.7 million cases annually.[1] In India, *Plasmodium vivax* is the most common species causing malaria (55% of all cases); however, *Plasmodium falciparum* is the main cause of malaria-related deaths.[2] To reduce morbidity and mortality associated with malaria, it is important that healthcare workers diagnose malaria quickly and accurately. Equally important is to avoid irrational use of antimalarial drugs in patients with an acute febrile illness, most of whom do not have malaria.[3] In areas of low transmission such as India, treatments based on identification of the species can help initiate species-specific treatments for *P. vivax* and *P. falciparum* malaria,[1] a

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strategy that can also reduce the development of drug resistance.[4]

India has an extensive programme for diagnosing malaria based on microscopic examination of peripheral blood films: more than 100 million slides are examined by microscopy annually.[2] Although malaria microscopy is the best and least expensive tool to diagnose malaria, it is beset with technical limitations such as poor quality of peripheral films, non-availability of equipment or skilled microscopists, and long turnaround times. Concerns regarding the accuracy of diagnosis by microscopy and the correctness of treatments in most malaria-endemic regions have meant that patients with fever are overdiagnosed as having malaria and are overtreated with antimalarial drugs.[5,6]

Rapid diagnostic tests (RDT) for malaria were developed to overcome these technical limitations and to supplement microscopy as the primary diagnostic modality in malaria-endemic countries. RDTs identify *Plasmodium* antigens using immunochromatographic techniques and their performance is influenced by the target antigen used, the incidence of malaria, the degree of parasitaemia and the predominant *Plasmodium* spp. in the region.[7,8] The earliest antigen target used in commercial RDTs was histidine-rich protein-2, specific to the asexual stages and young gametocytes of *P. falciparum* (PfHRP2).[9] Most PfHRP2-based tests have been shown to have a high accuracy [pooled sensitivity 92.7% (95% CI 91.0—94.5%) and pooled specificity 99.2% (95% CI 98.2—99.9%)].[8] Subsequently, *Plasmodium* lactate dehydrogenase (pLDH), which is conserved across all *Plasmodium* spp., was used as a target antigen.[10,11] The pLDH-based RDTs not only had lower accuracy estimates [pooled sensitivity 67.1% (95% CI 62.8—71.3%) and pooled specificity 98.4% (95% CI 97.5-99.6%)][8] but were unable to distinguish between the different *Plasmodium* spp.

Isolation of *P. vivax*-specific LDH (PvLDH)[12,13] heralded the development of diagnostic kits specific to the identification of *P. vivax* malaria. New RDTs that combine PfHRP2 and PvLDH have the potential to identify the commonest species causing malaria (*P. vivax*) from its more sinister counterpart *P. falciparum*. Because *P. vivax* and *P. falciparum* differ from each other in terms of disease severity, complications, management and prognosis, healthcare workers should be able to diagnose malaria with accuracy as well as identify the species correctly. This cross-sectional hospital-based study was designed to evaluate the diagnostic accuracy of a PfHRP2- and PvLDH-based RDT in a low-endemicity mixed infection area in rural central India, using malaria microscopy as a reference standard.

2. Methods

2.1. Setting

The Mahatma Gandhi Institute of Medical Sciences, Sevagram, is a rural-based teaching hospital located in Central India. In 2007, approximately 37 000 patients were admitted to this 660-bed hospital, 4900 (13.2%) of whom were assigned an infectious disease diagnosis on discharge. Approximately three-quarters of all patients with an infectious disease diagnosis were treated in the internal medicine wards of the hospital (unpublished data, hospital information system). Typically, the residents and internal medicine faculty evaluate all patients presenting in the outpatient and emergency departments of the hospital and patients with more severe symptoms are admitted to the internal medicine wards. Most infectious disease admissions are during the months of May and September, approximately one-half of whom are assigned a diagnosis of acute undifferentiated fever.

2.2. Patients

All consecutive patients aged $\geq$12 years admitted with a history of fever of $\leq$14 days duration to the medicine inpatient department and who were considered by their treating physicians for a malaria test were included. No exclusion criteria were employed. Patient age, gender, nature and duration of symptoms, and history of prior antimalarial therapy were recorded. Patients were followed until discharge from hospital and data were collected on clinically important outcomes. Written informed consent was obtained from all eligible patients.

2.3. Index tests

A commercially available, lateral flow, multispecies RDT kit (FalciVax; Orchid Biomedical Laboratories, Goa, India) that detects PfHRP2 and PvLDH was used. The index test was performed according to the manufacturer's instructions. The test strip is packaged in a cassette that has separate wells to deposit the sample and buffer solution. A disposable loop is used to transfer 5 μL of fingerprick whole blood to the sample well. Five drops of buffer are added and the sample flows through the conjugate pad to the testing window where the monoclonal antibodies anti-*P. falciparum* HRP2 (anti-PfHRP2 mAb), anti-*P. vivax*-specific PvLDH (anti-PvLDH mAb) and anti-rabbit IgM (control) are impregnated. The test is read at 15 min. Appearance of the control band alone indicates a negative test, whilst bands in the anti-PfHRP2 mAb and anti-PvLDH mAb regions indicate *P. falciparum* and *P. vivax* infections, respectively. Detection of PfHRP2 and PvLDH was considered as two separate index tests, both being reported as either positive or negative.

Before the study began, internal medicine residents and interns were trained to perform and interpret the RDT. Test kits were stored at 4 °C and a minimal delay was ensured between ordering the RDT and testing patients at the point of care. Patients' initials and test results were marked on the cassette during performance of the test. The results were verified by a study investigator (SW) and recorded on the electronic hospital information system. The test results were available within 30 min to the treating physicians for clinical decision-making.

2.4. Reference standard

Light microscopic examination of Giemsa-stained thick and thin peripheral blood films was used as a reference standard. The study peripheral blood films (or research slides) were drawn by the two residents (MM and SW) from fin-

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gerprick blood samples at the same time as the RDT was done to ensure that no time elapsed between the performance of the two tests and that the reference standard is performed before the treating physicians start their patients on antimalarial therapy. In addition to the research slides, non-research slides were also made and 2 ml of venous blood was withdrawn to obtain complete blood counts by an automated cell counter (Coulter AcT diff 2 haematology analyser; Beckman Coulter Inc., Fullerton, CA, USA). Results of these non-research blood tests were also available to the treating physicians for clinical decision-making.

A trained laboratory technician collected the slides, removed any patient identifiers from them and coded the slides. Peripheral films were stained with 10% Giemsa for 10 min by the same laboratory technician to ensure consistency. The films were examined by two trained pathologists (DJ and NG, with 6 years and 20 years of experience, respectively), who were blind to the results of the RDT and any clinical data. Discrepancy between the two pathologists was resolved by consensus. The pathologists examined at least 200 consecutive high-power fields (1000×) to classify the slide and paid attention to the morphological features of the parasite on the thin film to classify the species. They counted the number of trophozoites, schizonts and gametocytes in the positive blood films per 200 white blood cells. The parasite density per μL of blood was calculated from the white blood cell counts obtained by the automated Coulter counter.

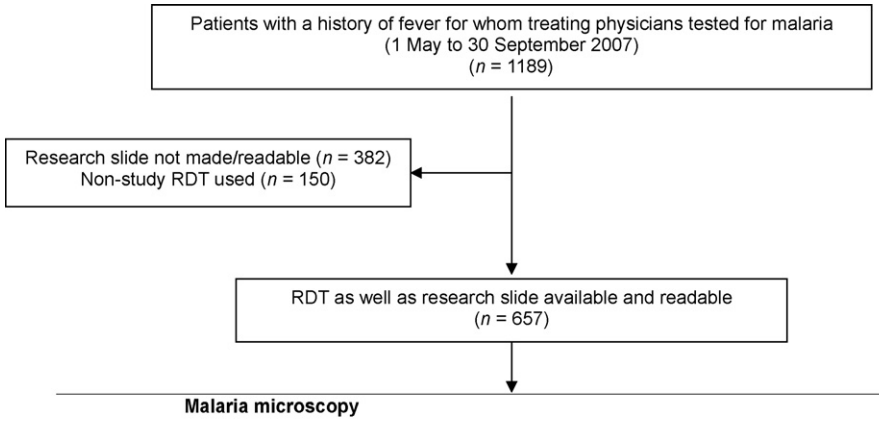
2.5. Statistical analysis

A descriptive statistical analysis was performed to compare the demographic and haematological variables in patients with and without malaria as well as to compare the characteristics of those whose microscopy and RDT differed in classifying the species. Student's *t*-test and χ^2 test were used as the tests of significance for the continuous and categorical variables, respectively.

Two-by-two tables were created to calculate point estimates and 95% CIs for the sensitivity, specificity, positive and negative likelihood ratios, and positive and negative predictive values of the RDT. Malaria microscopy was considered as the reference standard to estimate diagnostic properties of the index test. Results were analysed to answer three questions. First, what is the diagnostic accuracy of the RDT to detect malaria? Second, what is the diagnostic accuracy of the RDT to identify the malaria species? And third, what is the diagnostic accuracy of the RDT assuming that no misclassification in identifying the disease occurred? The Standards for Reporting of Diagnostic Accuracy (STARD) statement was used to describe the design of the study and the flow of patients.[14]

3. Results

Between 1 May and 30 September 2007, physicians ordered 1189 blood tests for malaria for patients admitted with acute



		Malaria microscopy				
		P. vivax	*P. falciparum*	*P. vivax and P. falciparum*	Negative	Total (%)
Rapid test	PvLDH for *P. vivax*	22 (3.3)	6 (0.91)	0	6 (0.91)	34 (5.2)
	PfHRP2 for *P. falciparum*	1 (0.15)	34 (5.2)	2 (0.30)	3 (0.46)	40 (6.1)
	PvLDH for *P. vivax and* PfHRP2 for *P. falciparum*	0	1 (0.15)	0	0	1 (0.15)
	Negative	4 (0.61)	1 (0.15)	0	577 (87.8)	582 (88.6)
	Total	27 (4.1)	42 (6.4)	2 (0.30)	586 (89.2)	657 (100)

All values indicate no. (%) of all cases (*n* = 657).

Figure 1 Study flowchart. RDT: rapid diagnostic test; PvLDH: *Plasmodium vivax*-specific lactate dehydrogenase; PfHRP2: *P. falciparum* histidine-rich protein-2.

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febrile illness. A total of 532 patients (45%) were excluded from the study either because their research slide was not made or readable or because a non-study RDT was used. The study sample consisted of the remaining 657 patients (55%) for whom results of the RDT and the research slide were available (Figure 1). The prevalence of malaria did not differ between those who were included in the study and those who were excluded (10.9% and 12.1%, respectively; $P = 0.49$).

The study patients ($n = 657$) were young (mean age 35.2 years, SD 17.0 years) and on an average had history of fever of 7.7 days (SD 11.3 days, range 1—14 days). Malaria microscopy was positive in 71 patients (10.8%) and negative in 586 (89.2%). RDT results were positive in 75 patients (11.4%) and negative in 582 (88.6%). Overall, 643 patients (97.9%) had concordant results by RDT and malaria microscopy [66 (10%) concordant positive and 577 (87.8%) concordant negative]. Compared with the patients with concordant negative test results, the patients with concordant positive tests were more likely to report rigors and had lower platelet counts (Table 1). Results of the RDT and microscopy for detection of a malarial parasite did not match in 14 patients (2.1%) (test discordance), of whom 9 (1.4%) had a positive RDT but negative microscopy. Only two of these nine patients had received antimalarial drugs prior to testing. The remaining five patients with discordant test results had a negative RDT and positive microscopy (parasite density 672—1440/μl) (Supplementary Table 1). One of these patients had received antimalarial drugs before testing. The overall sensitivity and specificity of the RDT to identify any parasite species were 92.9% (95% CI 85.0—97.3%) and 98.4% (95% CI 97.2—99.2%), respectively. Among all the febrile patients who tested positive, 87.1% truly had malaria, whilst of all those who tested negative 99.2% were truly negative (Table 2).

In nine patients (1.4%), the malaria species detected on the RDT differed from the one identified by microscopy (species discordance). In five patients the RDT detected antigen of *P. vivax* whilst microscopy detected trophozoites of *P. falciparum*, and in one patient the RDT detected antigens of *P. falciparum* whilst microscopy showed trophozoites of *P. vivax*. In three patients infected with both species, the RDT failed to pick up *P. vivax* in two (Supplementary Table 1). The positive predictive value (PPV) of the RDT to identify the species correctly was lower (74.5%, 95% CI 65.1—82.1%) compared with its PPV to detect any *Plasmodium* spp. (87.1%, 95% CI 77.8—92.8%). The sensitivities of PfHRP2 (for detection of *P. falciparum*) and PvLDH (for detection of *P. vivax*) antigens for specific species detection were lower than the combined estimates (Table 2).

Assuming that the RDT had correctly identified the species and that microscopy had erred, we corrected our diagnostic accuracy estimates. The corrected sensitivity and specificity of PfHRP2 for detection of *P. falciparum* were 95.0% (95% CI 84.4—99.1%) and 99.5% (95% CI 98.6—99.8%), respectively. The accuracy of the PvLDH test to detect *P. vivax* was lower [sensitivity 81.8% (95% CI 65.9—92.2%), specificity 98.7% (95% CI 97.5—99.4%)] (Table 2). The low sensitivity of the PvLDH test was not explained by a low parasite count (Supplementary Table 2).

4. Discussion

In this hospital-based, cross-sectional study it was observed that the PfHRP2/PvLDH test was 92.9% (95% CI 85.0—97.3%) sensitive and 98.4% (95% CI 97.2—99.2%) specific for all *Plasmodium* spp. The sensitivity and specificity estimates of the RDT for accurate species identification were 91.8% (95% CI 80.3—96.3%) and 96.8% (95% CI 94.5—97.8%). respectively. After correcting the data for species misclassification, the accuracy of the PvLDH test for detection of *P. vivax* remained low, whilst the PfHRP2 test for detection of *P. falciparum* performed as well as the estimates available from other studies.[8]

The patients in our study resemble those who would receive the test in actual clinical practice. The prevalence of malaria was similar between those who were included in the study and those who were excluded. The peripheral films were drawn at the same time as performance of the RDT to ensure that antimalarial therapy did not distort the reference standard. The index test and the reference standard were performed in all patients irrespective of the test results. Lastly, we chose the most appropriate reference standard for diagnosing malaria and ensured that it was interpreted by two experienced pathologists blind to the results of the RDT.

Our study has a few limitations. First, the study participants were hospital-admitted patients who are likely to have a more severe disease with high levels of parasitaemia, conditions in which the test is more likely to perform favourably. RDTs fail to pick up malaria when the parasite count is low, particularly those of *P. vivax*.[15] We do not know whether the test would perform as well in primary care or outpatient settings where parasitaemia is likely to be lower. Second, microscopy is an imperfect reference standard. We do not know whether microscopy-negative but RDT-positive patients were genuinely missed by microscopy or were incorrectly detected by RDT. Had we used more sensitive tests such as PCR, we could have possibly detected parasites in the peripheral films as low as 1—20/μl. However, our hospital does not offer PCR for diagnosing malaria. Third, pathologists find it hard to classify the *Plasmodium* sp. when peripheral films lack gametocytes or schizonts. Although *P. vivax* is often misclassified as *P. ovale*, failure to differentiate *P. falciparum* and *P. vivax* is also frequent[16] but underreported.[17] Zaman et al.[18] suggested that species misclassification often occurs when the parasite count is low, when infections are mixed and when only ring forms of the parasite are present. To accommodate the limitations of microscopy in misclassifying *Plasmodium* spp., a sensitivity analysis was performed assuming that the RDT had correctly identified the species. Lastly, we share the concern that exposure of malaria RDT kits to high temperatures may influence their performance.[19] Sevagram gets very hot (temperatures exceed 45 °C) in summer and most hospital wards are not air-conditioned. Although all kits were stored at 4 °C, it was not possible to avoid their exposure to temperatures >30 °C consistently during the point-of-care testing in hospital wards, a fact that could have affected the performance of PvLDH-based tests.

Both microscopy[20,21] and RDTs[22] have limitations in the diagnosis of mixed malarial infections. Previous studies from

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Table 1 Clinical and haematological characteristics of the study patients[a]

Characteristic	All patients	Both RDT and microscopy negative [Neg]	Both RDT and microscopy positive for *Plasmodium* sp. [Pos]	Discordance between RDT and microscopy [Dis]	*P*-value		
					[Pos] vs. [Neg]	[Dis] vs. [Neg]	[Dis] vs. [Pos]
Demographic and clinical characteristics (all patients)							
No.	657	577	66	14			
Age (years)[b]	35.2 (17.0) [12–90]	35.4 (17.2) [12–90]	35.2 (16.3) [14–70]	30.3 (14.6) [17–74]	0.99	0.22	0.22
Female sex	295 (44.9)	256 (44.3)	31 (46.9)	8 (57.1)	0.68	0.34	0.48
Duration of fever (days)[b]	7.7 (11.3) [1–14]	7.8 (9.6) [1–14]	7.9 (21.8) [1–14]	3.1 (1.8) [1–7]	0.96	0.06	0.41
Rigor	360 (54.8)	294 (51.4)	56 (86.1)	10 (71.4)	<0.01	0.13	0.17
Icterus	78 (11.9)	66 (11.5)	11 (16.6)	1 (7.1)	0.61	0.22	0.36
Altered behaviour	134 (20.4)	114 (19.8)	19 (28.7)	1 (7.1)	0.09	0.23	0.08
Haemoglobin (g/dl)[b]	12.1 (2.2) [5.4–15.1]	12.2 (1.9) [9.0–15.1]	10.9 (2.9) [5.4–11.2]	11.1 (3.3) [5.1–12.3]	0.05	0.13	0.12
WBC count ($\times 10^3/mm^3$)[b]	12.9 (46.5) [1.4–24.7]	13.8 (50.5) [1.4–22.0]	7.5 (3.4) [2.1–24.7]	8.3 (1.8) [3.2–16.4]	0.30	0.70	0.40
Platelet count ($\times 10^3/mm^3$)[b]	225.5 (280.4) [5–889]	240.2 (296.0) [5–600]	119.2 (86.0) [17–374]	167.7 (90.7) [40–320]	<0.01	0.41	0.09
Parasite count (microscopy-positive patients)							
Positive on microscopy	71	0	66	5			
Mean parasite count (asexual) (/μl)[b]	14693 (45391) [0–273600]	NA	15730 (46940) [0–273600]	1000 (319) [672–1440]			0.48
Mean parasite count (sexual) (/μl)[b]	239 (838) [0–5226]	NA	257 (867) [0–5226]	0			0.51
Mean parasite count (total) (/μl)[b]	14932 (45433) [4–274740]	NA	15987 (46977) [4–274740]	1000 (319) [672–1440]			0.48

RDT: rapid diagnostic test; WBC: white blood cell; NA: not applicable.
[a] All data indicate *n* (%) except where indicated.
[b] Mean (SD) [numbers in square brackets indicate range for continuous variables].

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Table 2 Diagnostic accuracy of the rapid diagnostic test (RDT) versus malaria microscopy as a reference standard

RDT vs. microscopy category	TP	FP	FN	TN	Sensitivity	Specificity	PPV	NPV	LR+	LR−
Detection of any *Plasmodium* spp.[a]	66	9	5	577	92.9 (85.0–97.3)	98.4 (97.2–99.2)	87.1 (77.8–92.8)	99.2 (98.1–99.6)	60.5 (31.5–116.1)	0.07 (0.03–0.17)
Correct identification of *Plasmodium* spp.[b]	56	19	5	577	91.8 (80.3–96.3)	96.8 (94.5–97.8)	74.5 (65.1–82.1)	99.1 (98.1–99.6)	28.8 (18.4–45.1)	0.08 (0.04–0.20)
Estimates based on species identification results as reported on microscopy										
Detection of *P. falciparum*	37	4	6	610	86.0 (73.2–94.1)	99.3 (98.4–99.8)	93.6 (84.5–97.5)	98.4 (96.8–99.2)	132.1 (49.3–353.4)	0.14 (0.07–0.30)
Detection of *P. vivax*	23	12	7	615	76.6 (59.2–89.1)	98.1 (96.7–98.9)	81.6 (71.1–88.9)	97.4 (95.2–98.6)	40.1 (22.1–72.6)	0.24 (0.12–0.46)
Estimates, if no misclassification occurred in species identification on microscopy[c]										
Detection of *P. falciparum*	38	3	2	614	95.0 (84.4–99.1)	99.5 (98.6–99.8)	95.6 (87.5–98.5)	99.4 (97.8–99.8)	195.4 (63.0–605.5)	0.05 (0.01–0.19)
Detection of *P. vivax*	27	8	6	616	81.8 (65.9–92.2)	98.7 (97.5–99.4)	87.6 (77.7–93.5)	97.9 (95.9–99.0)	63.8 (31.5–129.4)	0.18 (0.09–0.38)

TP: true positive; FP: false positive; FN: false negative; TN: true negative; PPV: positive predictive value; NPV: negative predictive value; LR+: positive likelihood ratio; LR−: negative likelihood ratio.

[a] No species distinction was made either on microscopy or on the RDT.

[b] The RDT was considered positive only if the same species was identified on microscopy. Discordant results (including all species misclassifications) were considered as negative.

[c] Microscopy results were revised assuming that the species identified by RDT was correct.

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India have reported a low incidence of mixed infection[22] and this is true even in other areas co-endemic for the two *Plasmodium* spp.[23,24] In this study, none of the three patients with mixed infection diagnosed by either test (malaria microscopy or RDT) could be confirmed by the other. In two cases where microscopy detected both species, the RDT could only detect *P. falciparum*.

Global sales of malaria RDTs are thought to surpass 70 million tests per year. From a single manufacturer in 1993, there are now many diagnostics companies manufacturing or rebranding malaria RDTs.[25] Of approximately 50 RDTs listed by the WHO, only four PvLDH antigen-based tests are commercially available that distinguish between *P. falciparum* and *P. vivax*.[26] Only three previous studies[23,27,28] have reported the accuracy of RDTs to differentiate *P. falciparum* from *P. vivax*. Forney et al.[23] evaluated the ParaSight F+V kit in Thailand, which had a high diagnostic accuracy for *P. falciparum* (sensitivity 98%, specificity 93%) but poor accuracy for *P. vivax* (sensitivity and specificity both 87%). This test is no longer marketed.[8] Bharti et al.[27] tested a Rapid Combo kit in India and found a reasonable accuracy for *P. falciparum* (sensitivity 93%, specificity 85%) but poor accuracy for *P. vivax* (sensitivity 83%, specificity 94%). Only one study conducted in the Amazon forests of Venezuela has reported the diagnostic accuracy of the FalciVax kit evaluated here. This study[28] reported an overall sensitivity of 36.4% and specificity of 99%. Two-thirds of malaria-positive patients in the study had a low parasite density (<100/μl), which could have led to the low sensitivity of the test. None of the patients with *P. vivax* infection in our study had a parasite density <200/μl.

RDTs for malaria were developed to overcome the limitations of microscopy and to reduce irrational treatment of acute febrile illnesses. They have revolutionised malaria diagnostics, have made it possible for healthcare workers as well as patients actually to see the test results and have brought malaria testing to the point of care.[19] Moreover, unlike malaria microscopy, these tests are not operator-dependent. To be useful they must achieve >95% sensitivity,[29] a target that has been achieved by PfHRP2-based tests but not by any of the rapid tests for non-*falciparum* species. Most RDTs that distinguish between *Plasmodium* spp. (such as BinaxNOW ICT[30] and OptiMAL[31]) aim to detect PfHRP2 and pLDH or aldolase as antigen targets. The pLDH-based products do not identify specific *Plasmodium* spp. and, in a meta-analysis[8] of six studies from malaria-endemic countries, the pooled sensitivity was lower (67%, 95% CI 62.8—71.3%) than estimates for PvLDH in our study. The poor sensitivity of tests detecting non-*falciparum* infection is of concern in low malaria endemicity countries like India where *P. vivax* infection is more common and other competing causes of fever co-exist. Another area of concern is the quality of the marketed RDTs. Malaria RDTs, virtually all of them listed on the WHO and UNICEF websites, need an external and independent quality control assessment. The first round of testing of malaria RDTs against a bank of highly characterised reference materials established at the CDC (Atlanta, GA, USA) is likely to be released soon. These results are expected to provide a head-to-head comparison between different test kits under rigorous testing conditions.[25]

The product insert of the RDT used here claims to have 100% sensitivity and specificity for the detection both of *P. falciparum* as well as *P. vivax* infections. This claim is based on an in-house study of 207 samples with known reference standard results.[32] However, our study conducted under actual field conditions gives a much lower sensitivity estimate for detection of *P. vivax* infection (76.6%) but a comparable sensitivity estimate for detection of *P. falciparum* (86%). Although this RDT has been designed to detect both *P. falciparum* and *P. vivax* infections and is being marketed as a species-specific combination test, our data show that compared with malaria microscopy, the RDT fails to pass this test largely owing to false-positive and false-negative test results with PvLDH-based *P. vivax* detection. Our results suggest that compared with malaria microscopy, the study RDT is specific but not sensitive. In light of an ongoing debate over the possibility of replacing field microscopy with RDTs, we recommend that as of now it would be premature to do so in regions where *P. vivax* infection is more common. Better antigen targets for diagnosis of *P. vivax* infection by RDTs need to be identified.

Authors' contributions: All authors were involved in designing the study; MM and SW conducted and reported the rapid diagnostic tests; DJ and NG performed malaria microscopy; RJ, SS and SPK analysed and interpreted the results. All authors contributed to the preparation of the article, provided critical revisions and approved the final version. SPK is guarantor of the paper.

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Ethical approval: The study was approved by the Institutional Review Board at Mahatma Gandhi Institute of Medical Sciences, Sevagram, India.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.trstmh.2009.04.011.

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PLOS ONE

Asymptomatic *Plasmodium falciparum* Malaria in Pregnant Women in the Chittagong Hill Districts of Bangladesh

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Abstract

Background: Pregnancy is a known risk factor for malaria which is associated with increased maternal and infant mortality and morbidity in areas of moderate-high malaria transmission intensity where *Plasmodium falciparum* predominates. The nature and impact of malaria, however, is not well understood in pregnant women residing in areas of low, unstable malaria transmission where *P. falciparum* and *P. vivax* co-exist.

Methods: A large longitudinal active surveillance study of malaria was conducted in the Chittagong Hill Districts of Bangladesh. Over 32 months in 2010–2013, the period prevalence of asymptomatic *P. falciparum* infections was assessed by rapid diagnostic test and blood smear and compared among men, non-pregnant women and pregnant women. A subset of samples was tested for infection by PCR. Hemoglobin was assessed. Independent risk factors for malaria infection were determined using a multivariate logistic regression model.

Results: Total of 34 asymptomatic *P. falciparum* infections were detected by RDT/smear from 3,110 tests. The period prevalence of asymptomatic *P. falciparum* infection in pregnant women was 2.3%, compared to 0.5% in non-pregnant women and 0.9% in men. All RDT/smear positive samples that were tested by PCR were PCR-positive, and PCR detected additional 35 infections that were RDT/smear negative. In a multivariate logistic regression analysis, pregnant women had 5.4-fold higher odds of infection as compared to non-pregnant women. Malaria-positive pregnant women, though asymptomatic, had statistically lower hemoglobin than those without malaria or pregnancy. Asymptomatic malaria was found to be evenly distributed across space and time, in contrast to symptomatic infections which tend to cluster.

Conclusion: Pregnancy is a risk factor for asymptomatic *P. falciparum* infection in the Chittagong Hill Districts of Bangladesh, and pregnancy and malaria interact to heighten the effect of each on hemoglobin. The even distribution of asymptomatic malaria, without temporal and spatial clustering, may have critical implications for malaria elimination strategies.

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Background

Pregnant women are at greater risk of acquiring malaria infection and developing symptomatic and complicated malaria disease than their non-pregnant counterparts [1]. Malaria infection during pregnancy, with or without symptoms, is a known cause of maternal anemia [1–4]. Massive sequestration of *Plasmodium falciparum* parasites in the placenta, with or without detectable parasites in the peripheral circulation, is a distinct feature of pregnancy-associated malaria [5,6], and is believed to be responsible for an increased risk of adverse pregnancy outcomes including miscarriage, stillbirth, prematurity, and the delivery of a low birth-weight baby [2,6–8].

The major risk factors associated with malaria during pregnancy include young maternal age, a low number of previous pregnancies (primi- or secundigravidae), and gestational age in pregnancy [9]. In addition, limited data suggest that pregnancy-associated malaria may play a critical role in pre-eclampsia [10–12], a serious pregnancy-associated disorder associated with negative pregnancy outcomes with worsened maternal and infant

survival. In African countries with moderate to high malaria transmission, antimalarial drug combination sulfadoxine-pyrimethamine is used to protect pregnant women, regardless of malaria symptoms, from the malaria-related negative pregnancy outcomes [13]. This treatment, known as intermittent preventive treatment of malaria in pregnancy (IPTp), significantly reduces malaria-related adverse effects on the mother and the fetus and improves birth outcomes [14,15] and has been implemented as part of routine antenatal care in 34 of 44 African countries with ongoing malaria transmission following the recommendations of World Health Organization.

Much of the knowledge about pregnancy-associated malaria is primarily based on data from sub-Saharan Africa where malaria transmission intensity is moderate to high and *P. falciparum* dominates. Data on the nature and magnitude of malaria on pregnant women and their pregnancy outcomes is relatively sparse in regions where malaria transmission intensity is low or unstable and mixed infections with *P. falciparum* and *P. vivax* are common [16]. It is generally postulated that malaria infection in such hypoendemic settings, defined as transmission in the regions where less than 10% of 2–9 year old children are positive for malaria parasites and where malaria tends to manifest itself as seasonal outbreaks, is more likely to be symptomatic compared to infections in hyperendemic settings due to lack of meaningful immunity to malaria parasites [17]. However, evidence suggests that pregnant women in hypoendemic regions may suffer from more severe and complicated disease [18], and that asymptomatic infections may be more common in the pregnant populations than expected [19].

We conducted a field clinical longitudinal study to assess whether pregnancy is a risk factor for asymptomatic *P. falciparum* malaria in the Chittagong Hill Districts (CHD) of Bangladesh. In Bangladesh, 26.9 million people residing in 13 of its 64 districts are at risk of malaria and approximately 50,000 clinical malaria cases and 100–500 malaria-related deaths were reported annually (unpublished data, Bangladesh Ministry of Health and Family Welfare, 2009). The highest incidence of malaria is found in the CHD [20,21], a remote, forested ecozone located in Bangladesh's southeastern region contiguous with Myanmar that is primarily inhibited by 12 non-Bengali ethnic tribal groups [22]. *P. falciparum* malaria transmission is considered hypoendemic in the region, since the incidence rates were estimated as 1.48 and 2.75 per 1,000 person-months in 6–59 months and 5–14 years old, respectively [23]. Our calculated incidence rate for children age 2–9 years old is 1.53 infections per 1,000 person-months (roughly equivalent to the infection rate 1–2% of tested population per year). Symptomatic cases clustered geographically and seasonally, with the highest case load in rainy season in May-October when 80% of cases were identified [23]. Previous observations in a large malaria survey of the population suggest that the rates of asymptomatic malaria may be 2–3 times higher than the symptomatic rates (unpublished data, BRAC). This paper describes a clinical field study to estimate the period prevalence of asymptomatic malaria infection among apparently healthy pregnant women, in comparison with non-pregnant women and adult men.

Methods

Study design, site and population

This was a clinical longitudinal field surveillance study to assess if pregnancy is a risk factor for asymptomatic *P. falciparum* malaria. The study was conducted as part of a large passive and active malaria surveillance system established in the CHD of Bangladesh to study the epidemiology of malaria in the region, and to link demographic, clinical, and entomologic factors in the determination of important risk factors for malaria. The details of this study system and its methods of data collection have been published elsewhere [24]. Briefly, demographic and malaria surveillance was conducted in two demographically defined unions (Kuhalong and Rajbila) of the CHD, covering a population of approximately 24,000 individuals in 4,500 households over a defined area of 179 km^2 (Figure 1). The region was divided into 24 study clusters in the two unions for programmatic purposes with each cluster containing approximately 1,000 individuals. Initial demographic and socioeconomic data were collected from October 2009 to February 2010 in Kuhalong and from April to August 2010 in Rajbila. Immigration, emigration, births and deaths of the study population were monitored and updated by trained surveillance workers every 3–4 months.

Passive and active malaria surveillance was initiated in Kuhalong union in October 2009 and Rajbila union in April 2010 by trained field workers. Data from active surveillance between May 2010 and January 2013 (total of 32 months of active surveillance) were included in this analysis. Active surveillance was conducted as cross-sectional, random screening of the population using a two-stage cluster sampling design, as previously described [24]. For the non-pregnant adult and children populations, 12 individuals per union were selected for active malaria screening per week stratified by age, with four individuals from each pre-specified age groups ($<$5 years, 5–14 years, $\geq$15 years). In each union, two of the 12 individuals were randomly selected and entered into a longitudinal surveillance cohort and tested for malaria every three months. Pregnant women were continually identified in the study area through the demographic surveillance system by field workers at the village level or through a pregnancy test for women selected into active surveillance. Selection of pregnant participants was independent of the study design for the passive and active surveillance in the non-pregnant populations. All pregnant women identified in the two study unions were invited to join the study. All consenting pregnant women were enrolled, placed in the longitudinal surveillance cohort, and tested for malaria every three months until delivery. After delivery, the pregnant women were counted as postpartum (within 6 months after delivery) and non-pregnant (after 6 months postpartum) and were continued to be tested for malaria every three months in the longitudinal surveillance cohort.

Individuals were tested for asymptomatic malaria infection by a rapid diagnostic test (RDT) using FalciVax test strip, which detects both *P. falciparum* and *P. vivax* malaria, and microscopic examination of thick and thin blood smears. The reported sensitivity and specificity of FalciVax RDT were relatively high (97.6% and 95.8% for *P. falciparum*, and 76.5% and 100% for *P. vivax*, respectively) in febrile patients who typically present with a medium-high parasite density [25]. However the detection ability of the RDT may be lower in asymptomatic parasitemic individuals where lower parasite density is expected [26]. Asymptomatic malaria infection was defined as a positive RDT or blood smear for *P. falciparum* in the absence of any clinical signs or symptoms suggestive of malaria, and only asymptomatic infections were included in this analysis. *P. vivax* infections were not included in this analysis because only one asymptomatic P. vivax infection was detected in the study population during the time period of the analysis. The presence of symptoms was subjectively investigated by general open-ended and specific questions, and body temperature was measured. Finger prick blood was collected for RDT and smear reading, and for retrospective PCR analysis. A positive infection was defined if either or both an RDT or blood smear was positive. A standard protocol for malaria microscopy quality management established at the International Center for Diarrhea

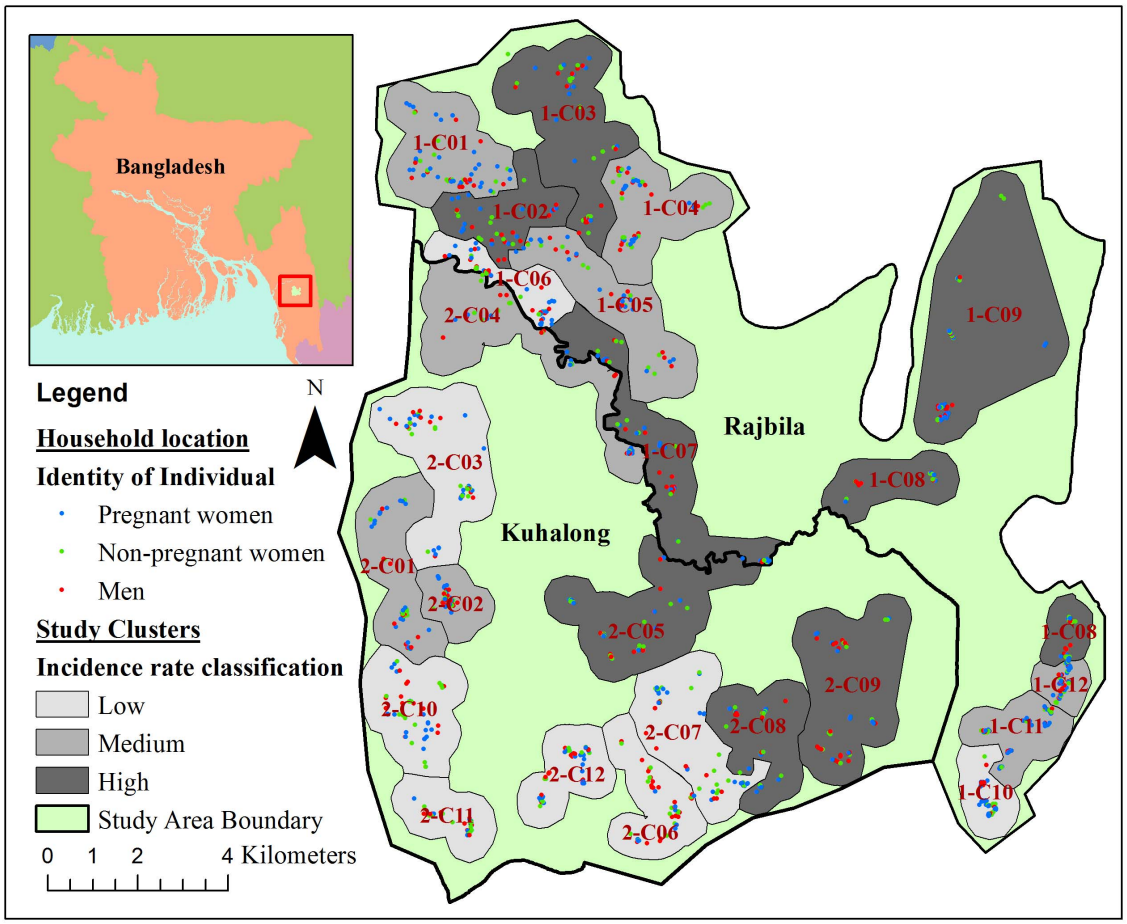

Figure 1. ArcGIS spatial analytical map for active surveillance of asymptomatic malaria. Red, green or blue colored dots represent the household locations of participating men, non-pregnant women, or pregnant women, respectively. Background dark, medium or light grey color represents the highest, medium or lowest eight incidence clusters, respectively.
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Disease Research, Bangladesh (icddr,b) was followed, where a random sample of 100 positive and 100 negative samples were examined and independently validated by two microscopists not associated with the study. Positive RDT or blood smear, regardless of clinical symptoms, prompted a treatment with a standard three-day course of artemether-lumefantrine. Pregnant women positive for malaria during the first trimester were treated with quinine as per national treatment guidelines [13], although this event was rare. The first day of treatment was designated as day 0. Follow up of confirmed malaria took place on days 2, 7 and 28. At each visit, blood was collected and examined by smear microscopy to ensure the resolution of infection.

Detailed description of DNA extraction and real-time PCR (rt-PCR) analysis is available elsewhere [27]. Briefly, five 3 mm diameter punches, equivalent to 25 µl of whole blood, were removed from the filter papers. DNA was extracted with a commercial 96- well kit (Qiagen Mini Blood DNA extraction kit cat. no. 51106) and concentrated with a glycogen-acetate and acetate/ethanol precipitation and low-speed (3,000 g) centrifuge for 30 minutes. Multiplex rt-PCR amplified the 18S *P. falciparum* ribosomal gene with a Cy5 labeled probe. Samples were run in duplicate on manually loaded 96-well plates. A 40 cycle standard PCR protocol was run on a BioRad CFX96 PCR Detection System (BioRad, Hercules, CA, USA). Baseline relative fluorescence unit (RFU) values were readjusted within the Bio-Rad CFX manager software. The rt-PCR method demonstrated the highest sensitivity in the latent class analysis in febrile patients, and was able to detect 1–100 parasites per microliter. PCR testing was conducted for study samples collected between May 2010 and March 2012. Blood samples from non-endemic patients at Johns Hopkins University were used as negative control. Blood was also collected from the enrolled pregnant and non-pregnant women in the longitudinal surveillance, for the measurement of hemoglobin concentration using the hemocue system (Hemocue Hb 201, Hemocue America). Testing was performed in all women who were enrolled in the study from July 2011 to the end of the study.

Household locations for all individuals tested for malaria infection were mapped to show the spatial distribution of malaria tests in the study area. GPS coordinates for households were collected by local surveillance workers in March-April 2009, and mapped in ArcGIS (version 10.1; ESRI, Redlands USA). Using these GPS coordinates and passive malaria surveillance data, incidence clusters were defined based on the symptomatic *P. falciparum* incidence rates determined in a previous analysis [23]. These 24 study clusters were divided into three incidence categories containing eight clusters each labeled as "high incidence", "medium incidence", and "low incidence".

Statistical analysis

This study was designed to estimate the period prevalence of asymptomatic *P. falciparum* infection among pregnant women, non-pregnant women and adult men from May 2010 to January 2013, and investigated the relationship between asymptomatic malaria infection and the following risk factors over the same time period: pregnancy status, study union, the symptomatic malaria incidence of the study cluster, age, ethnicity, occupation, bed net use, and transmission season. Asymptomatic malaria infection was defined as a positive blood smear or RDT for *P. falciparum* malaria without any clinical signs or symptoms suggestive of malaria. The analysis was restricted to asymptomatic individuals 16–44 years old who were tested at least once by active surveillance between May 2010 and January 2013 and had complete lab data. Only day 0 tests were included in this analysis, and tests on follow-up days 2, 7 and 28 were excluded.

Age was stratified into two groups, those at or above the median age (25 years) and those below the median age. Anemia was defined as hemoglobin concentration less than 11.0 g/dL. Mild, moderate or severe anemia was defined as hemoglobin concentrations 10–10.9 g/dL, 7–9.9 g/dL, or less than 7 g/dL, respectively. Occupations of interest included housewives, agriculture, daily labor, jhum cultivation, and others (mostly students and unemployed). Jhum cultivation is a form of migrant agriculture in the hilly CHD region and has been implicated as a risk factor for symptomatic *P. falciparum* infection in this study area [23]. The temporality of asymptomatic infection was explored based on known seasonal transmission patterns: November to April as the low transmission season and May to October as the high transmission season. Other binary variables of interest in this analysis included study union (Rajbila or Kuhalong), ethnicity (non-tribal Bengali or tribal groups), and bed net use the previous night (yes or no). Study clusters were categorized as high, medium, and low incidence based on symptomatic *P. falciparum* incidence rates as described in the Methods section previously (Figure 1). Non-pregnant women were classified as postpartum if a test was taken within six months of the estimated date of delivery documented while she was pregnant (a proxy for the birth date) or non-postpartum if the test was taken later than six months after the estimated date of delivery or the women had never been pregnant.

The sociodemographic and temporal factors were compared between pregnant women, non-pregnant women and men ages 16–44 years using a Chi^2 test. The period prevalence of asymptomatic malaria infection between pregnant women, non-pregnant women and men as well as within each of these risk categories were calculated and assessed for statistically significant differences using a Chi^2 test. The period prevalence was calculated for both postpartum non-pregnant women and non-postpartum, non-pregnant women and compared using a Chi^2 test.

The independent effect of pregnancy and other factors on asymptomatic *P. falciparum* infection was determined using multivariate logistic regression analysis. Dummy variables were created for demographic and temporal factors of interest and used to calculate the univariate and multivariate associations between these factors and asymptomatic malaria infection. Because very few asymptomatic *P. falciparum* cases occurred in the eight low incidence clusters, only data from the sixteen medium and high incidence clusters were used to maximize statistical power. The final multivariate logistic regression model was selected based using forward and backward stepwise regression (covariates were selected using a likelihood ratio test; $p<0.1$) after assessing the interactions between covariates. Models were assessed for fit using Hosmer-Lemeshow and Pearson's Chi^2 goodness of fit tests and checked for collinearity by calculating variance inflation factors.

The effect of asymptomatic malaria infection and pregnancy status on blood hemoglobin level was assessed. The median and inter-quartile range of blood hemoglobin concentration (in g/dL) were calculated and presented as box plots for asymptomatic malaria cases and non-cases, pregnant women and non-pregnant women, and the interaction of the two. Statistical significance was determined using a non-parametric Kruskall-Wallis equality of populations test. The percent of each group that was moderately or severely anemic (<10 g/dL) was calculated for each group and assess for statistical significance using a Chi^2 test. All statistical analyses were conducted in Stata (version 12.1; Statacorps, College Station, USA).

The overall period prevalence of asymptomatic malaria infection detected by PCR was calculated for pregnant women, non-pregnant women and men, and stratified into RDT- or smear-positive and PCR-positive infections that were negative by RDT or smear.

Ethical considerations

A written informed consent or assent was obtained prior to enrollment from all adult or children (as defined as less than 16 years of age) participants, respectively. Permission from a guardian was also obtained for children. Additional consent was obtained at the time of enrollment into the active surveillance cohort. The study protocol and informed consent form were approved by the icddr,b Ethical Review Committee (PR#09021) and the Johns Hopkins Bloomberg School of Public Health Institutional Review Board (IRB#1965). The study was conducted in accordance with the principles of research ethics stated in the Declaration of Helsinki and the local and international regulatory guidelines.

Results

Study population characteristics

A total of 3,110 tests of malaria RDT and blood smear from individuals ages 16–44 years old were performed as part of active surveillance from May 2010 to January 2013. Among these, 909 tests were taken from 526 pregnant women, 1,753 tests from 911 non-pregnant women, and 448 tests from 316 men. Tests gathered from active surveillance were distributed evenly across the study area for men, pregnant women and non-pregnant women (Figure 1).

The demographics of these different groups are described in Table 1. Pregnant women were more likely to be from Rajbila, younger (61% of pregnant women were 16–24 years old compared to 44% and 31% of non-pregnant women and men, respectively), and more likely to be tested during the high transmission season (62% of pregnant women compared to 56% and 55% in non-pregnant women and men, respectively). Pregnant women, non-pregnant women and men also had significantly different occupation patterns and tended to live in different study clusters when study clusters were stratified by symptomatic *P. falciparum* incidence rates. There were no significant differences observed in ethnicity and bed net use among the three groups.

Risk factors for asymptomatic *P. falciparum* infection

The period prevalence of asymptomatic *P. falciparum* infection was assessed by calculating the percentage of tests that were positive for *P. falciparum* malaria infection by a RDT or blood smear microscopy from May 2010 to January 2013 (Table 2). There were a total of 34 asymptomatic infections (21 pregnant women, 9 non-pregnant women and 4 men), originated from 34

Table 1. Description of the study participants.

Demographic factors	Pregnant (%)	Non-pregnant (%)	Men (%)
Union			
Rajbila	538 (59.2)	872 (49.7)	219 (48.8)
Kuhalong	371 (40.8)	881 (50.3)	229 (51.1)
Study Incidence cluster			
Low Incidence	269 (29.6)	629 (35.9)	156 (34.8)
Medium Incidence	371 (40.8)	616 (35.1)	140 (31.3)
High Incidence	269 (29.6)	508 (29.0)	152 (33.9)
Median age (25 years)			
$<$ median age (16–24)	554 (61.0)	769 (43.9)	139 (31.0)
$\geq$ median age (25–44)	355 (39.1)	984 (56.1)	309 (69.0)
Ethnicity			
Bengali	175 (19.3)	331 (18.9)	85 (19.1)
Tribal	734 (80.8)	1,422 (81.1)	359 (80.1)
Occupation			
Housewife	399 (45.6)	669 (38.4)	1 (0.2)
Agriculture	175 (20.0)	425 (24.4)	162 (36.5)
Daily labor	76 (8.7)	159 (9.1)	83 (18.7)
Jhum cultivation	109 (12.4)	267 (15.3)	72 (16.2)
Other	117 (13.4)	221 (12.7)	126 (28.4)
Bed net use			
Yes	577 (89.2)	1,372 (91.1)	384 (88.5)
No	70 (10.8)	134 (8.9)	50 (11.5)
Transmission season			
High (May-August)	560 (61.6)	978 (55.8)	247 (55.1)
Low (Sept-April)	349 (38.4)	775 (44.2)	201 (44.9)
Total	909	1,753	448

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different participants. All 34 infections were RDT-positive and 27 of them were both RDT- and smear-positive. The median number (range) of tests per participant across study populations was 2 (1–4), 2 (1–7) and 1 (1–4) in pregnant women, non-pregnant adult women and adult men, respectively, and 43.9% (231/526), 44.7% (407/911), and 83.2% (263/316), of pregnant women, non-pregnant adult women and adult men were tested only once. 19 of 21 (90.5%), 3 of 9 (35%) and 3 of 4 (75%) of positive infections in the pregnant, non-pregnant and male population, respectively, were tested positive on the first testing. There were no recurrent infections after treatment or no repeated infection in the same individual. Pregnant women had the highest period prevalence of asymptomatic *P. falciparum* infection detected by RDT or smear (2.3%) compared to non-pregnant women (0.5%) and men (0.9%) (Chi^2 test; $p<0.001$). Statistically significant risk factors for asymptomatic *P. falciparum* infection included residence in Rajbila union (1.6% compared to 0.5% in Kuhalong union), and younger age (1.7% in those 16–24 years old compared to 0.6% in those 25–44 years). Individuals living in study clusters of high symptomatic *P. falciparum* incidence had the highest period prevalence of asymptomatic infection (1.8%) compared to study clusters of medium (1.1%) and low incidence (0.5%). There were no significant differences in the period prevalence of different occupation groups, among those reporting bed net use the previous night compared to those who did not, and across tests taken in the high and low transmission seasons. Among non-pregnant women, there was no statistically significant difference between the period prevalence of asymptomatic malaria among those tests taken $\leq$6 months postpartum (4 cases among 800 tests; 0.5%) and those taken from women who have never been pregnant or $>$6 months postpartum (5 cases among 953 tests; 0.5%).

Results of the PCR analysis were available for the first 2,362 (716 samples from 454 pregnant women, 1,242 from 690 non-pregnant women and 404 from 308 men) blood samples, collected from May 2010 to March 2012. Of these, a total of 67 samples (2.8%) were PCR-positive: 33 (4.6%), 23 (1.9%) and 11 (2.7%) in pregnant women, non-pregnant women, and men, respectively. Of these PCR-positive samples, 32 were also positive by RDT/smear. Therefore the PCR detected additional 35 infections that were negative by RDT or smear: 14 (2.0%), 14 (1.1%) and 7 (1.7%) samples in pregnant women, non-pregnant women, and men, respectively.

The independent effect of pregnancy on asymptomatic *P. falciparum* infection

The unadjusted and adjusted odds of asymptomatic *P. falciparum* infection were calculated using univariate and multivariate logistic regression modeling (Table 3). Only data from study clusters of high or medium symptomatic *P. falciparum* incidence were used for this analysis (Figure 1). After using stepwise selections to generate a

Table 2. Prevalence of asymptomatic malaria infection by demographics.

Demographic factors	Total	Malaria positive (%)	*p*-value
Respondent tested			
Pregnant women	909	21 (2.3)	<0.001
Non-pregnant women	1,753	9 (0.5)	
Men	448	4 (0.9)	
Union			
Rajbila	1,629	26 (1.6)	0.005
Kuhalong	1,481	8 (0.5)	
Study incidence cluster			
Low Incidence	1,054	5 (0.5)	0.015
Medium Incidence	1,127	12 (1.1)	
High Incidence	929	17 (1.8)	
Median age (25 years)			
< median age (16–24)	1,648	25 (1.7)	0.002
≥ median age (25–44)	1,462	9 (0.6)	
Ethnicity			
Bengali	591	2 (0.3)	0.050
Tribal	2,515	32 (1.3)	
Occupation			
Housewife	1,069	10 (0.9)	0.107
Agriculture	762	6 (0.8)	
Daily labor	318	1 (0.3)	
Jhum cultivation	448	8 (1.8)	
Other	464	9 (1.9)	
Bed net use			
Yes	2,333	18 (0.8)	0.490
No	254	3 (1.2)	
Transmission season			
High (May to August)	1,785	22 (1.2)	0.386
Low (September to April)	1,325	12 (0.9)	
Total	3,110	34 (1.1)	

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final multivariate logistic regression mode that accounts for the inter-relatedness of the final covariates, pregnant women had 5.4-fold higher odds of asymptomatic *P. falciparum* infection as compared to non-pregnant women (p = 0.001). Men had 2.5-fold higher odds of infection than non-pregnant women, however this relationship was not statistically significant (p = 0.214). Other factors selected into the final model included living in Rajbila union as compared to Kuhalong union (OR = 2.8; p = 0.072) and age, with the odds of infection decreasing by 15% per year of age (OR = 0.85; p = 0.001). The final model was assessed for goodness-of-fit and found to adequately fit the data (Pearson's $Chi^2 = 113.9$, p = 0.995; Hosmer-Lemeshow $Chi^2 = 10.7$, p = 0.221).

The association between pregnancy and asymptomatic *P. falciparum* infection with blood hemoglobin

Hemoglobin concentrations were available in 495 (54.5%) and 444 (25.3%) pregnant and non-pregnant women, respectively. The median and interquartile range of blood hemoglobin concentration was calculated and stratified by pregnancy status and asymptomatic malaria infection status (Figure 2). Overall, the median blood hemoglobin concentration was lower in pregnant than non-pregnant women (10.0 g/dL *vs.* 10.7 g/dL), and in women with than without asymptomatic falciparum infection (8.3 g/dL *vs.* 10.4 g/dL). The presence of both pregnancy and malaria appeared to interact and magnify the negative impact, as pregnant women with asymptomatic *P. falciparum* malaria had the lowest median blood hemoglobin (8.0 g/dL) compared to women with non-pregnant, malaria negative women (10.7 g/dL), non-pregnant, malaria positive women (10.8 g/dL), and pregnant, malaria-negative women (10.0 g/dL). A greater proportion of pregnant than non-pregnant women (51.5% *vs.* 33.3%, Chi^2 test p<0.001) and malaria positive than malaria negative women (84.6% *vs.* 42.3%, Chi^2 test p = 0.002) had moderate or severe anemia (hemoglobin less than 10 g/dL). Of 21 malaria positive pregnant women, 10 were tested for hemoglobin, and all were moderately to severely anemic. Of 9 malaria-positive non-pregnant women, 3 were tested for hemoglobin and 1 was moderately to severely anemic.

Table 3. Odds of asymptomatic infection in high & medium incidence clusters.

	Unadjusted		Adjusted	
Demographic factors	**OR (95% CI)**	***p*-value**	**OR (95% CI)**	***p*-value**
Respondent tested				
Non-pregnant women	1.0		1.0	
Men	2.3 (0.6–9.8)	0.250	2.5 (0.6–10.6)	0.215
Pregnant women	7.6 (2.8–20.2)	<0.001	5.4 (2.0–14.5)	0.001
Union				
Kuhalong	1.0		1.0	
Rajbila	3.6 (1.2–10.2)	0.019	2.7 (0.9–7.8)	0.071
Age (continuous)	0.83 (0.76–0.91)	<0.001	0.85 (0.77–0.93)	0.001
Ethnicity				
Bengali	1.0		Not selected[b]	
Tribal	3.4 (0.5–25.4)	0.225		
Occupation (N = 2021)[a]				
Agriculture	1.0			
Housewife	1.4 (0.4–4.5)	0.622	Not selected[b]	
Daily labor	0.5 (0.1–4.6)	0.551		
Jhum cultivation	2.2 (0.6–7.6)	0.211		
Other	3.1 (1.0–10.3)	0.059		
Bed net use (N = 1,672)[a]				
No	1.0			
Yes	0.6 (0.2–2.2)	0.450	Not selected[b]	
Transmission season				
Low (Sept. to April)	1.0			
High (May to August)	1.3 (0.6–2.7)	0.555	Not selected[b]	

OR, odds ratio; 95% CI, 95% confidence interval;
[a]Missing data in demographic and bednet survey;
[b]Not selected into final multivariate model based on higher than 10% Type I error on LR test.
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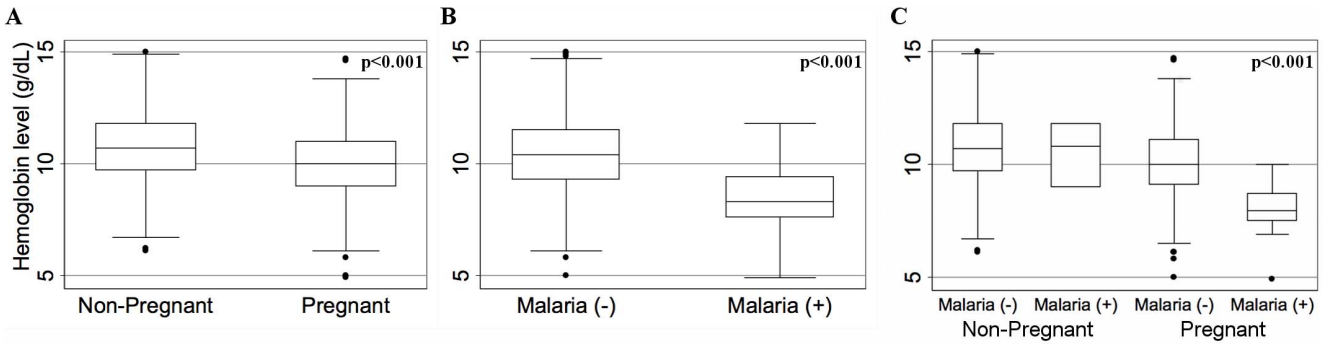

Figure 2. Box-plots of hemoglobin in pregnant and non-pregnant women with or without asymptomatic malaria. Box-plots represent the median and interquartile range of hemoglobin concentration. Panel **A** shows values in non-pregnant (n = 444) and pregnant (n = 495), **B**, malaria negative (n = 926) and malaria positive (n = 13), and **C**, the interaction of pregnancy and malaria indicating the lowest hemoglobin concentration in malaria-positive pregnant women (n = 10) compared with malaria-negative non-pregnant (n = 441) or pregnant (n = 485) women or malaria-positive non-pregnant women (n = 3). (Statistical significance by non-parametric Kruskal-Wallis equality of populations test)
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Discussion

In this study of asymptomatic *P. falciparum* malaria in the Chittagong Hill Districts of Bangladesh, pregnant women had significantly higher period prevalence of asymptomatic *P. falciparum* infection compared to men and non-pregnant women over 32 months of active and longitudinal malaria surveillance. Using a real time PCR analysis we detected a similar ratio of malaria prevalence between pregnant women, non-pregnant women, and men, as compared to RDT or blood smear. RDT or smear detected about one-half of infections detected by rt-PCR. None of the submicroscopic infections detected by PCR progressed to clinical malaria.

This association held true in a multivariate logistic regression model controlling for maternal age and study union in the 16 study clusters with the highest malaria incidence. These results are consistent with other studies from high-transmission settings in sub-Saharan Africa [1] and among limited evidence from low-transmission settings [2,28,29] that show that pregnant women are at greater risk for *P. falciparum* infection.

This study shows two important findings. First, the temporal and spatial distributions of asymptomatic infections differ from that of symptomatic disease. These observations were made over a similar time period, excluding the possibility of changes in transmission over time potentially confounding the observed infections. We previously found that the incidence of symptomatic *P. falciparum* infection in all age groups was 6-fold higher in the high transmission season (May-October) than the low transmission season (November-April), and 18.3-fold higher in the high incidence cluster than in the low incidence clusters [23]. In this study, however, there was no significant difference in the period prevalence of asymptomatic *P. falciparum* infection between the two transmission seasons. While a subsequent analysis will investigate the epidemiology of asymptomatic infection in all age groups in this study area, our study results suggest that asymptomatic malaria infection is more evenly distributed across time and space than symptomatic infections. This observation will have a critical implication in the development of strategies for malaria control, particularly in the era of malaria elimination in regions such as Bangladesh where low transmission intensity justifies such an attempt. Further research is urgently needed to understand the nature and distribution of asymptomatic malaria infection serving as an important infected reservoir to continue malaria transmission.

Second, our observation in this study also suggested that *P. falciparum* infection and pregnancy synergistically contribute to maternal anemia in a hypoendemic malaria setting. Either pregnancy or malaria infection was associated with low hemoglobin concentration, and the women who were both pregnant and malaria positive had the lowest hemoglobin concentration. While hemoglobin values were available only in a portion of women, and the number of malaria-positive was too low to draw a definitive conclusion, the results are in agreement with evidence from high transmission settings [3,7,30–33], and support the limited evidence in low transmission settings [2,34,35], with one study in Ethiopia showing that malaria-positive pregnant women were more likely to be anemic in a low compared to a high transmission area [36].

While this study was grossly underpowered to detect the negative health consequences related to malaria during pregnancy, our findings echoed previous observations of detrimental effects of malaria in pregnant women and the fetus, and highlighted an urgent need to optimize the management of asymptomatic malaria infection in pregnant women residing in hypoendemic settings. While the risk-benefit balance may not favor of the use of IPTp, a mass preventive malaria treatment to minimize negative consequences of malaria in pregnant women widely used in regions of sub-Saharan Africa with moderate to high malaria transmission, screening of all pregnant women using RDT at prenatal visits may be effective. Further research is warranted to assess the effects of malaria detection and treatment targeting among this complex and vulnerable group of women.

The reported rate of the use of bed nets (ITN) was high in our study, but we did not systematically measure the effectiveness of, or the lack thereof, ITNs in our study populations. The use of ITNs is one of the strategies that has been shown in other studies to improve malaria control and childhood mortality and morbidity [37] and provide protection for pregnant women [38]. These findings should be interpreted with caution since methodological and statistical flaws have been reported [37] and some studies have reported paradoxical findings that increased ITN use is associated with increased malaria incidence in the face of ITN distribution [39]. Data on ITN effectiveness are sparse in low transmission countries such as Bangladesh, and are critically needed to examine the impact of ITN on asymptomatic infection as well as anemia in pregnant women of such settings.

There are a number of important limitations to this study. First, we performed repeated testing in the same individual and the effect of non-independent measurement may have systematically

biased our results. However, for this analysis, we assume that data points are independent since all infections originated from independent participants and the majority of positive infections were positive on the first testing. It is, therefore, unlikely that our study finding can be explained by a systematic error of repeated testing, or by the disproportionate testing frequency in one study population over the other. Similarly, this assumption of independence among repeated testing in the same individual may not be true since previous malaria infection may provide some immunity to subsequent infections. However, the likelihood that this assumption biased the results in this hypoendemic setting is low. Second, the study is underpowered with only 34 asymptomatic *P. falciparum* infections, including only two infections in non-tribal Bengalis. These low numbers could not accommodate a multivariate model to assess potentially important factors risking overstratification of the data and leading the large confidence intervals. Therefore, important confounders and effect modifiers of the effect of pregnancy on malaria infection may be missing from the model. Despite this limitation, this study was still powered enough to detect a clinically relevant 2–4 fold increase in the period prevalence of *P. falciparum* infection in pregnant women compared to non-pregnant women, adjusted for age and study location. Third, there is potential discrepancy between the estimated date of delivery and actual date of pregnancy termination, raising a risk of incorrect assignment of some women who had miscarriage or premature delivery. However we were able to confirm the date of pregnancy termination in 28 of 30 women, using information on the delivery outcome in the demographic surveillance system, and their status of pregnancy at the time of the detection of asymptomatic malaria infection. Moreover we were able to measure hemoglobin only in a portion of study women, and did not examine the association between asymptomatic malaria risk and gestational age of pregnancy, gravidity or parity, or pregnancy outcomes, for which a larger stratified study will be needed. Similarly, we did not address the impact of pregnancy on *P. vivax* infection, an important known cause of pregnancy anemia and maternal and infant morbidity [35], or symptomatic malaria disease. Further work is needed to identify the relationship between *P. vivax* infection and pregnancy status in Bangladesh, especially in areas where *P. vivax* is more common.

Conclusion

In summary, this study shows that the risk of asymptomatic *P. falciparum* infection is significantly higher in pregnant women than in non-pregnant adult women or men in the Chittagong Hills Districts of Bangladesh, and suggests that the presence of *P. falciparum* infection in pregnant women may heighten the risk of anemia. These results underscore the need to further evaluate ways to optimize management of malaria infection in pregnant women living in areas where malaria transmission intensity is low. Management of asymptomatic infections at a population level, particularly in a low transmission setting, is complex owing to the risk-benefit ratio as well as to its social acceptance. Pregnant women present a different and important argument due to the known risk of malaria, regardless of symptomatic illness, to the mother and fetus. While argument for mass treatment such as IPTp widely used in high-transmission settings in sub-Saharan Africa may not be favorable in areas of low malaria transmission intensity such as Bangladesh, active screening and prompt treatment, with or without clinical symptoms, should be considered for high-risk populations. Our study also shows that asymptomatic *P. falciparum* infections, the most critical and problematic parasite population in breaking the transmission cycle, are evenly distributed throughout time and space, differing from the observed pattern of symptomatic infections which tend to cluster geographically and with time. These findings have critical implications for malaria control or elimination strategies. Further research is warranted to understand the distribution of asymptomatic infected reservoir, and to assess ways to eradicate it at the population level.

Author Contributions

Conceived and designed the experiments: WAK MR GG DEN TS DAS DJS MMN. Performed the experiments: WAK SRG CSP JK SA MR MSA MZH JA GG DEN TS DAS DJS MMN. Analyzed the data: WAK SRG MR SA MZH JA GG DEN TS DAS DJS MMN. Contributed reagents/materials/analysis tools: DAS DJS SRG MR GG TS DEN MMN. Wrote the paper: WAK SRG DAS DJS MMN. Participated in the data analyses and interpretation, preparation of this manuscript, and final approval: WAK SRG CSP JK SA MR MSA MZH JA GG DEN TS DAS DJS MMN.

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The role of age, ethnicity and environmental factors in modulating malaria risk in Rajasthali, Bangladesh

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Abstract

Background: Malaria is endemic in the Rajasthali region of the Chittagong Hill Tracts in Bangladesh and the Rajasthali region is the most endemic area of Bangladesh. Quantifying the role of environmental and socio-economic factors in the local spatial patterns of malaria endemicity can contribute to successful malaria control and elimination. This study aimed to investigate the role of environmental factors on malaria risk in Rajasthali and to quantify the geographical clustering in malaria risk unaccounted by these factors.

Method: A total of 4,200 (78.9%; N = 5,322) households were targeted in Rajasthali in July, 2009, and 1,400 individuals were screened using a rapid diagnostic test (Falci-vax). These data were linked to environmental and socio-economic data in a geographical information system. To describe the association between environmental factors and malaria risk, a generalized linear mixed model approach was utilized. The study investigated the role of environmental factors on malaria risk by calculating their population-attributable fractions (PAF), and used residual semivariograms to quantify the geographical clustering in malaria risk unaccounted by these factors.

Results: Overall malaria prevalence was 11.7%. Out of 5,322 households, 44.12% households were living in areas with malaria prevalence of ≥ 10%. The results from statistical analysis showed that age, ethnicity, proximity to forest, household density, and elevation were significantly and positively correlated with the malaria risk and PAF estimation. The highest PAF of malaria prevalence was 47.7% for third tertile (n = 467) of forest cover, 17.6% for second tertile (n = 467) of forest cover and 19.9% for household density >1,000.

Conclusion: Targeting of malaria health interventions at small spatial scales in Bangladesh should consider the social and socio-economic risk factors identified as well as alternative methods for improving equity of access to interventions across whole communities.

Background

Malaria eradication is the ultimate goal of the World Health Organization (WHO) [1]. Most malaria endemic countries are presently shifting their efforts from malaria control to eradication [2]. The WHO has targeted eight to ten countries for elimination of malaria by 2015 and afterwards in all other endemic countries [1]. Although the long-term goal is world-wide eradication, there remains some debate over the feasibility of elimination in Africa and eleven Asian malaria endemic countries [2-5]. Accurate maps of malaria incidence are important tools in malaria control as they can guide interventions and assess their effectiveness. Risk maps and geo-spatial data are increasingly used to support malaria elimination [6].

The Bangladesh national malaria survey in 2007 indicated that malaria was endemic in 13 of 64 administrative districts and that the crude prevalence is 4.0% [7,8]. In Bangladesh, prevalence is very low across large parts of the country except in Chittagong Hill Tracts (CHT), where risk is especially high among marginalized and hard-to-reach communities [6]. Moderate-to-high risk

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remains in well-defined areas across Bangladesh [6]. Active case detection with rapid diagnosis tests (RDT) conducted in 2007 revealed that the total malaria prevalence in the CHT was 11.7% with a majority of infections (>90%) caused by *Plasmodium falciparum* [7]. One particular sub-district, Rajasthali, had a prevalence of 36% and is considered the area most endemic to malaria in CHT [7]. In 2009, another prevalence study carried out in Rajasthali found 11.5% of participants positive with malaria infection despite 2 years of an extensive intervention and control program supported by the Global Fund [9]. Malaria is transmitted in a broad range of eco-epidemiological settings in Bangladesh due to high species diversity and the presence of species displaying different ecological behaviors [10]. Successful malaria control and elimination in Bangladesh can only be achieved if the country can target efficiently the endemic foci. Most of these hotspots are situated in CHT, along the borders of India and Myanmar where 90% of total cases occurs [6-8,11]. These areas are remote, forested and populated by ethnic minorities living a traditional rural life. The vectors present in this forested area are *Anopheles baimai* (dirus), *Anopheles philippinensis, Anopheles vagus* and *Anopheles minimus* [10]. Malaria risk is related to environmental factors affecting these vectors including altitude, forest, household density, cultivation practices, urbanization, and distance from water bodies [9,11-13], but the role of these factors to malaria risk has not been quantified.

At present controlling malaria in Bangladesh is based on both preventing infections and on prompt effective treatment of clinical cases. Through the National Malaria Strategic Plan, the malaria and parasitic disease control unit supports malaria prevention and treatment services in 13 endemic districts of Bangladesh.

Predicting the abundance, as well as understanding the risk factors, spatial distribution, and spread of malaria in endemic settings can significantly contribute to local malaria control strategies. A deeper understanding of the role of landscape attributes in the spatial distribution of malaria is crucial so that appropriate local elimination efforts can be developed.

Malaria prevalence in Bangladesh shows a pronounced heterogeneity of transmission [6]. Models predict that unrecognized heterogeneity reduces the efficacy of disease control strategies [14]. While escalating control measures in disease hotspots may be very effective at reducing overall transmission. Recent efforts to develop pragmatic global maps, such as Malaria Atlas Project (MAP), suggests a new era of using maps to define regional populations at risk of malaria, that will guide future global malaria control and the distribution of funds [15]. The most modifiable cause of malaria risk is having two or fewer bed nets; non-modifiable risk factors are associated with sex, age, forest cover, elevation and household density [9]. The relative importance of a risk factor can be estimated using the PAF. The odds ratio and the relative risk can only measure the level of risk associated with the exposure to a risk factor. They never reflect the impact of the factor in a population. PAF is the proportional reduction in population disease that would occur if exposure to a risk factor were reduced over a specified time interval from the population. Many diseases are caused by multiple risk factors, and individual risk factors may interact in their impact on overall risk of disease. PAF estimates can provide some insight for policymakers in planning public health interventions. The goal of this study was to estimate the PAF for different environmental factors for malaria in Rajasthali that will generate key information as a new phase of malaria control in Bangladesh begins with the support of the Global Fund.

Methods

Study area

Covering a surface area of 145.04 km^2, Rajasthali lies between 22°20' to 22°26' north latitude and 92°08' to 92° 22' east longitude in CHT. It is a hilly, remote area covered with dense forest. The total population of Rajasthali is 24,097 with 5,322 households. Most households belong to ethnic clans leading a traditional rural life. All households (n = 5,322) in Rajasthali were georeferenced [16].

Malaria prevalence, socio-economic and environmental data

The malaria prevalence data used in this analysis was collected during a 2009 malaria-prevalence survey in Rajasthali using a two-stage cluster sampling technique. A questionnaire survey was conducted at the same time to obtain demographic information, bed net usage and socio economic status. A full description of the survey has been published [9]. Blood samples from 1,400 (> 0-102 years of age) individuals were tested using rapid diagnosis test. Out of 1,400 samples, 161 (11.5%) were positive for either *P. falciparum* (158, 11.3%) or *Plasmodium vivax* (11, 0.8%) [9]. Geometrically corrected, cloud free Landsat 5 Thematic Mapper image was taken on January 23rd, 2010 (Path 136, Row 44) from the United States Geological Survey. Based on the review of existing literature and considering the importance of land cover types, a supervised classification was performed. The study area was classified into six categories (deep water, shallow water, brown open land, bright open land, forest, and grassland/bush) based on the maximum likelihood method for PG-Steamer (Pixoneer Geomatics Inc., Tae-jon). Ground-truth sites and known land-cover were identified from high-resolution Google

Earth images. The proportion of forested area within 2 km from each of 1,400 sampled households was calculated using Arc GIS 9.3. Later the proportion of forested area was categorized into tertiles for the analysis. A Shuttle Rader Topographic Mission Digital Elevation Model (SRTM DEM) of 3 arc-second (approximately 90 m) resolution was used to estimate the altitude of each sampled household using ArcGIS 9.3. Local house density was also used for analysis. Data preparation and collation of these items has been described in detail [9].

Ethical consideration

This study was reviewed and approved by both the research review committee and ethical review committee of International Center for Diarrhoeal Diseases Research Bangladesh (ICDDR, B). Written consent was taken from study participants or their caretakers [9].

Data analysis

Assessing variables

All statistical analyses were carried out using the statistical software STATA 11 (Stata Corp., College Station, TX). Data included individual-level variables (Age, sex, tribe); household level variables (Household head education and occupation, number of bed nets, long lasting insecticidal net (LLIN) use, bed net treatment and household floor materials) and environmental variables (forest, altitude, household density). The statistical analysis was carried out in two phases using malaria infection status (based on rapid diagnostic test result) as the outcome variable of interest. Firstly, all individual, household and environmental variables were screened using univariate logistic regression for statistical association with malaria infection status based on a liberal p-value of 0.20 in the likelihood-ratio test. Secondly, all variables significant in the screening phase were considered for inclusion through a manual backward stepwise variable selection process in a multivariable logistic regression analysis. The criterion for removal of risk factors was based on statistical considerations using the likelihood ratio test with a significance level of $p > 0.05$. The variables age, sex, ethnicity, number of bed net, forest, altitude, household density and floor were retained for the final multivariate model

Assessing spatial autocorrelation

The semivariogram was used as a graphical representation of the spatial dependence in the data. The residuals of the final model (age, forest, altitude and household density) were extracted and screened for spatial dependence using semivariograms developed with the package geoR of the statistical software R version 2.9.0 (The R foundation for Statistical Computing, Vienna). The residuals of a model was also tested without the non-significant ($p > 0.1$) variables (sex, tribes, number of bed net, LLIN, bed net treatment, floor, household head education and occupation), to observe changes to the semivariograms.

Estimation of the population attributable fractions of malaria

The PAF of risk factors included in the final multivariable model was estimated using the AFLOGIT procedure of STATA [17]. PAF estimates the proportional amount of disease risk if all the risk factors were simultaneously eliminated from the population. The adjusted PAF highlights how much of the disease risk is attributed to all the factors included in the model. From the initial descriptive analysis, reference levels for each factor with the lowest risk were determined. That ensured that the PAFs were derived from positive associations with the outcome. A univariate analysis for each risk factor was performed. Then adjusted ORs, PAF and 95% CIs from multivariate models including all the statistically significant risk factors were reported.

Results

The spatial distribution of malaria prevalence in the 1,400 locations surveyed in Rajasthali shows that the distribution of malaria prevalence is heterogeneous across communities (Figure 1) ranging from 4.26% to 45.24% (Table 1). Out of 5322 households, 44.12% were in areas with malaria prevalence of ≥ 10%.

Age, sex, ethnic association, number of bed nets, forest, altitude, household density and floor type proved statistically significant in univariate analyses (Table 2). Only age, altitude, forest and household density proved statistically significant in the multivariate model.

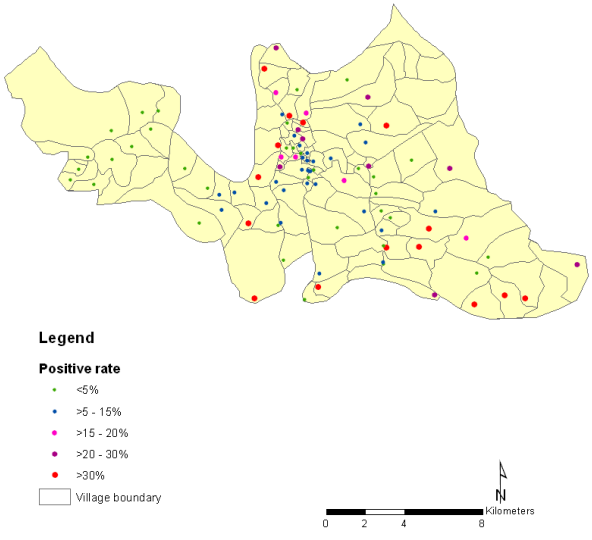

Figure 1 Spatial distribution of malaria prevalence in Rajasthali region, Bangladesh for 2009.

Table 1 Malaria prevalence distribution of households included in the 2009 malaria survey in the Rajasthali region, Bangladesh

Malaria prevalence	Number of households
4.26 - 8.92%	2912
> 8.92 - 14.22%	2213
> 14.22 - 20.65%	96
> 20.65 - 29.01%	79
> 29.01 - 45.24%	23

Children aged up to 10 years and people living in higher altitude areas had much higher odds of malaria infection than their peers. The odds of malaria increased with increasing elevation. People living around the densest forest (upper third tertile) were at a significantly higher risk of being infected compared to the first and second tertiles. In addition to age, altitude and forest, the odds of malaria also increased with increasing household density. In univariate analysis, lower household density (1-200) proved as the highest risk factor for infection. However, in the multivariate model, higher household density (>1000) produced an increased risk. Adjusted malaria risk with >1,000 households was significantly higher than for (1-200) households density (OR, 3.61 [95% CI, 1.34-9.74]; P = 0.011).

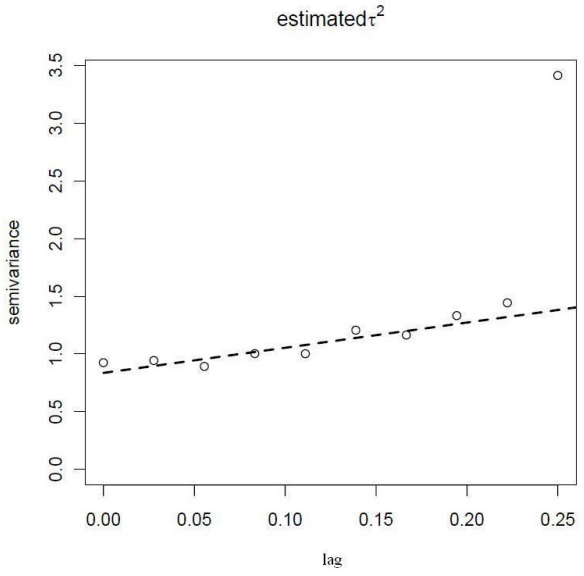

Figure 2 Residual semivariogram of malaria prevalence in Rajasthali using the final multivariable model.

After taking into account the effect of the individual- and survey-level environmental variables, there was little evidence for spatial dependence (Figure 2). For that reason, extra spatial parameters were not fitted to the regression.

Table 2 Results of unadjusted and adjusted logistic regression model for RDT positive status

Covariates	Unadjusted OR (95% CI)	*P* value	Adjusted OR* (95% CI)	*P* value
Ethnicity				
Tripura	1.00		1.00	
Bengali	3.55 (1.48-8.48)	0.004	2.19 (0.87-5.52)	0.096
Marma	3.36 (1.43-7.87)	0.005	2.30 (0.94-5.59)	0.066
Other	2.56 (1.04-6.29)	0.040	1.72 (0.67-4.43)	0.258
Altitude (meter)				
≤ 50	1.00		1.00	
51 - 100	1.57 (1.10-2.25)	0.013	1.54 (0.94-2.51)	0.084
> 100	5.27 (3.06-9.08)	0.000	3.82 (1.88-7.76)	0.000
Household density				
1 - 200	1.00		1.00	
201 - 500	0.46 (0.29-0.71)	0.001	1.57 (0.83-2.97)	0.167
501 - 1000	0.40 (0.20-0.78)	0.007	3.12 (1.14-8.57)	0.027
> 1000	0.37 (0.25-0.55)	0.000	3.61 (1.34-9.74)	0.011
Forest density				
1st Tertile (n = 466)	1.00		1.00	
2nd Tertile (n = 467)	2.21 (1.32-3.70)	0.003	2.66 (1.52-4.66)	0.001
3rd Tertile (n = 467)	4.62 (2.86-7.45)	0.000	9.02 (3.74-21.74)	0.000
Age				
>10 years	1.00		1.0	
0-10 years	2.75 (1.94-3.89)	0.000	2.60 (1.80-3.75)	0.000

*All variables included in the model

Table 3 Population Attributable Fraction of positive rapid test result for malaria associated with environmental risk factors

Covariates	Population Attributable fraction	95% CI
Tribe		
Tripura (reference)		
Bengali	14.5%	-0.6% - 27.3%
Marma	23.8%	1.1% -41.2%
Other	7.3%	-4.6% - 17.8%
Altitude (meter)		
≤ 50 (Reference)		
51 - 100	11.2%	-1.9% - 22.6%
> 100	9.3%	3.4% - 14.3%
Household density		
1 - 200 (Reference)		
201 - 500	6.5%	-2.7% - 14.9%
501 - 1000	4.4%	0.2% - 8.3%
> 1000	19.9%	8.6% -29.9%
Forest density		
1st Tertile (n = 466) (Reference)		
2nd Tertile (n = 467)	17.6%	7.8% - 26.3%
3rd Tertile (n = 467)	47.7%	36.1% - 57.2%

*estimated using the adjusted odds ratio

Population attributable fraction

The PAFs were estimated for the environmental covariates; altitude, population density and forest (Table 3). Adjusting for all other risk factors, the factors with the highest PAFs were the third tertile of forest cover (47.7%), being a member of the Marma community (23.8%) and living in a place where house density was > 1,000/sq km (19.9%). Most of the Marma villages are located in the western and central regions of Rajasthali. Household density is highest in the central area of Rajasthali where most of the administrative buildings are located. Forest density is higher in the eastern parts of Rajasthali. The second tertile of forest cover accounted for 17.6% of the PAF, Bengali people accounted for 14.5% and household elevation (altitude 51-100 m from sea level) accounted for 11.2% (Table 3). Other communities including Khiang and Chakma (7.3%) [16], and living at > 100 m altitude (9.3%) with lower household density (201-500) accounted for the 6.5% PAFs (Table 3).

Discussion

The purpose of this study was to estimate the PAFs for different contributors to malaria risk and to develop a local malaria risk model in a highly endemic area of Bangladesh. The results show that at local spatial scales there was little evidence to include environmental covariates (such as temperature and precipitation) in the models and only the covariates age, forest, altitude and household density were significantly associated with infection. Furthermore, there was little evidence for spatial dependence in semivariograms after the model was developed, suggesting little evidence of substantial unexplained variation that varied predictably over the region. Taken together these results suggest that locally, individual-level factors (e.g. socioeconomic factors; behavioral factors; adherence to preventative measures) are more likely to determine the spatial distribution of malaria infections. The dataset did not include additional individual-level variables describing behavior and further surveys aimed at developing spatial risk models at small spatial scales should endeavor to include this type of information. The inclusion of these individual-level variables may improve the discriminatory ability of the model but unfortunately were not available in the Rajasthali dataset. The greatest PAFs were altitude, household density and forest cover.

Presumably due to lower acquired immunity, children are more vulnerable for malaria infection [7,18-20]. Results from this study are consistent with the age related effects. The high PAFs for forest coverage and high altitude suggest a critical role of specific malaria vectors. In this region *An. dirus, An. minimus*, and a diverse fauna of other anopheline species have been reported as main malaria vectors, with *An. dirus spp* being an efficient vector in forests habitats [21,22]. Although the vector species have not been sampled in the study area, the higher risk of infection in hilly and forested area may implicate the primary vector species as *An. dirus*. Despite the small overall elevation distribution in Rajasthali (from 22 m to 359 m above the sea level), this factor may have some contribution to characterize differences of transmission risk, and malaria prevalence rate. Altitude proved one of the key factors responsible for malaria transmission in CHT [8]. Few studies have confirmed that elevation is one of the key factors associated with malaria [23-25]. Study results from Afghanistan showed no transmission in villages at elevations >2,000 m [26]. The altitude in all households of Rajasthali is < 360 m and, thus, not high enough to provide cooler temperatures.

Univariate analyses and PAF further revealed that lower household density was a significant risk factor for malaria infection. This pattern is similar to a study from

Ghana that reported higher malaria risk in smaller villages and in outer areas of each village [27]. However, for the multivariate model, the effect of household density was reversed and definitive conclusions could not easily be made. Strong correlations were observed between the forest coverage and house density, and may account for the results in the multivariate model.

The relative importance of risk factors for public health intervention can be estimated using PAF. PAF defines the reduction of incidence and can be achieved if the population had been entirely unexposed compared to its current exposure pattern [28]. The results indicate that altitude, household density and forest were the most important risk factors associated with malaria prevalence in the study area.

These findings are important for targeted intervention and resource allocation. The greatest use of PAFs here has highlighted modifiable risk factors, predicting how much disease can be avoided with their elimination. The non-modifiable factors cannot be eliminated here (e.g., ethnicity). Modifiable risk factors should be considered to prioritize and target public health intervention strategies in CHT. The different ethnic community living in the very remote regions (close to deep forest and in high altitude areas) accounted for the highest PAFs. These results suggest that if living conditions and access to treatment care services can improve, then it would be possible to prevent a significant proportion of malaria incidence in this population. PAF estimates obtained in this study will be useful since the malaria control programme in Bangladesh is enhancing its control efforts and has committed to reduce malaria cases by 60% by 2015 as the baseline year/cases of 2015 [29].

The increasing rate of malarial infection with greater distances from the village centre could also be due to proximity to mosquito breeding sites. Residences on the village periphery may be located closer to swamps and agricultural fields that result in a greater local mosquito density, although we have no data to indicate this occurred. Socioeconomic status can be protective against malaria if it provides better access to anti-malarial medicines or bed nets, which is the ultimate priority in accordance to malaria control program in Bangladesh. In the central and western areas, it is possible that the more established families with more resources live near the town centre or market [27]. These trends could also be explained by the proximity of health clinics and markets that sell bed nets and anti-malarial medications to the village centre. These findings are pivotal for national control programs and could help policy makers identify high-risk areas that can be targeted first (Figure 1). The calculations (Table 3) are then able to inform program managers if they need to re-distribute or recruit additional health workers to cover 100% of the population in this area. It has important implications in meeting the first key objective of the control programme, which is to effectively diagnosis and treat 100% of estimated malaria cases [29]. Targeting those foci with higher expected prevalence within forested areas will become particularly important for Bangladesh.

Conclusions

This study indicates that prevalence in Rajasthali remains high. Through identification of high-risk malaria zones one may generate new hypotheses regarding the spatial distribution of malaria. It can also be used as advocacy for channeling more funds to conduct operational research in high-risk areas. Targeting of interventions at such fine spatial scales will be helpful for the malaria control programme. Given the social and socio-economic risk factors are identified, more consideration could be given to methods of improving equity of access to interventions among whole communities in the region.

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Conflict of interest

The authors declare that they have no competing interests.

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Authors' contributions

Conceived the study design: UH, RJSM. Data preparation: UH. Data analysis: UH, DM. Prepared the manuscript: UH, GG. Conception, overall scientific management, interpretation of result, and critically reviewed the final report: KK, RH, RJSM, WP, GG. All authors approved the final version of the manuscript. Principal investigator of the project: UH.

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Knowledge on the transmission, prevention and treatment of malaria among two endemic populations of Bangladesh and their health-seeking behaviour

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Abstract

Background: Data on sociological and behavioural aspects of malaria, which is essential for an evidence-based design of prevention and control programmes, is lacking in Bangladesh. This paper attempts to fill this knowledge gap by using data from a population-based prevalence survey conducted during July to November 2007, in 13 endemic districts of Bangladesh.

Methods: A two-stage cluster sampling technique was used to select study respondents randomly from 30 *mauzas* in each district for the socio-behavioural inquiry (n = 9,750). A pre-tested, semi-structured questionnaire was used to collect data in face-to-face interview by trained interviewers, after obtaining informed consent.

Results: The overall malaria prevalence rate in the 13 endemic districts was found to be 3.1% by the Rapid Diagnostic Test 'FalciVax' (*P. falciparum* 2.73%, *P. vivax* 0.16% and mixed infection 0.19%), with highest concentration in the three hill districts (11%). Findings revealed superficial knowledge on malaria transmission, prevention and treatment by the respondents. Poverty and level of schooling were found as important determinants of malaria knowledge and practices. Allopathic treatment was uniformly advocated, but the 'know-do' gap became especially evident when in practice majority of the ill persons either did not seek any treatment (31%) or practiced self-treatment (12%). Of those who sought treatment, the majority went to the village doctors and drugstore salespeople (around 40%). Also, there was a delay beyond twenty-four hours in beginning treatment of malaria-like fever in more than half of the instances. In the survey, gender divide in knowledge and health-seeking behaviour was observed disfavouring women. There was also a geographical divide between the high endemic south-eastern area and the low-endemicnorth-eastern area, the former being disadvantaged with respect to different aspects of malaria studied.

Conclusion: The respondents in this study lacked comprehensive knowledge on different aspects of malaria, which was influenced by level of poverty and education. A gender and geographical divide in knowledge was observed disfavouring women and south-eastern area respectively. They preferred allopathic treatment for

malaria, although a substantial proportion did not seek any treatment or sought self-treatment for malaria-like fever. Delay in seeking care was common. The implications of these findings for programme development are discussed.

Background

Malaria is a public health problem in some ninety countries worldwide including Bangladesh and estimated to be responsible directly for about 3,000 deaths a day worldwide [1]. The poor and vulnerable populations are disproportionately affected by malaria and the severe consequences of malaria are borne more by the poorest [2]. There is also strong evidence that the use of preventive and treatment interventions for malaria depends upon socio-economic status (SES) [3-5]. The economic burden of ill health, such as malaria, on individual households can be substantial and in some cases catastrophic, especially for the poor households [6]. Prevention and control of malaria thus can contribute towards poverty alleviation efforts in Bangladesh.

In Bangladesh, the National Malaria Control and Prevention Programme is currently being implemented by the Malaria and Parasitic Disease Control (M&PDC) unit of the Directorate General of Health Services (DGHS), Government of Bangladesh [7,8]. M&PDC implements the programme in the community in partnership with a BRAC-led consortium of 20 small NGOs (selected through competitive bidding) under funding from GFATM round 6 [8]. BRAC, an indigenous micro-credit/micro-finance-based NGO, is working with the twin objectives of alleviation of poverty and empowerment of the poor [9]. As part of its efforts to mitigate the income-erosion consequences of illnesses for the poor households, BRAC is involved in malaria control activities in the three high endemic hill tracts districts since 1998. This current five-year programme (2007–2012) implemented in all of the 13 endemic districts has both preventive (distribution of LLIN/ITNs, intermittent insecticide residual spray and awareness building programmes) and curative (presumptive case management, early diagnosis and prompt treatment following WHO guidelines, and referral of complicated cases to tertiary facilities) components towards reducing malaria morbidity and mortality [7].

A baseline survey was done during the inception phase of the programme to estimate the parasitological prevalence of malaria infection and record benchmark information on the awareness and knowledge of the community regarding the transmission, prevention and treatment of malaria and relevant health-seeking behaviour. The data generated is expected to fill in the knowledge gaps in social science aspects of malaria in Bangladesh and help programme develop informed intervention components and strategies (by BRAC and other NGOs), and also, future programme evaluation and impact assessment [10].

Methods

Data for this paper originated from the baseline survey conducted during July to November 2007, to cover the peak malaria season in Bangladesh. The 13 malaria-endemic districts were divided into two groups based on endemicity [11]: the five high endemic (parasitic prevalence 7.2%) south-eastern districts (henceforth SE area), and the eight low endemic (parasitic prevalence 0.5%) north-eastern districts (henceforth NE area) (Figure 1). The districts are composed of *upazilas* (sub-districts) divided into Unions, the latter again divided into *mauzas* (lowest administrative unit equivalent to, but not necessarily equal to, villages).

Sampling

Two-stage cluster sampling technique was employed to select the study sample. City Corporations and towns were excluded from this survey. For each of the 13 districts, all *mauzas* were listed and 30 *mauzas* were selected using a probability proportional to size (PPS) sampling procedure [11]. These *mauzas* were the primary sampling unit. Twenty-five households were selected using systematic random sampling from each *mauza*. Sample size was calculated using web-based software (C-Survey 2.0) based on the conservative estimates of malaria prevalence of 2%, design effect of 2, and precision of 1.5% at 95% confidence interval. This yielded a sample size of 750 individuals in each district for the study (total = 9,750).

The survey

The survey consisted of two parts: parasitological prevalence estimation by RDT and socioeconomic survey related to malarial knowledge and relevant health-seeking behaviour. For the latter, a semi-structured questionnaire was developed and pre-tested for ascertaining consistency, appropriateness of language, and sequencing before finalization [see Additional file 1]. The survey team comprised of experienced social science graduates who received rigorous training for five days on questionnaire content, probing techniques and strategies to establish rapport and neutrality essential to complete and accurate data collection. In hilly areas interviewers from ethnic groups were recruited to interview respective ethnic group of people.

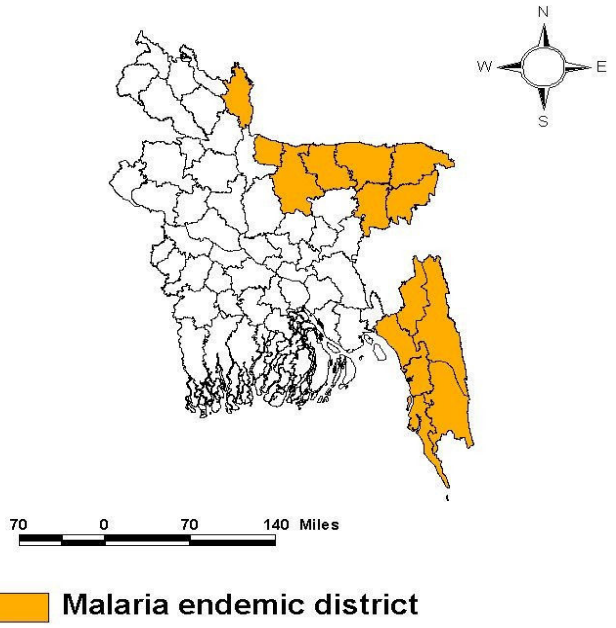

Figure 1
Two Malaria endemic areas of Bangladesh.

In each *mauza*/village, the survey team selected every third household encountered as they moved from the center or periphery of the *mauza*/village following a designated path using the "spin the bottle" methodology [12]. When there were no respondents present in the selected households or the respondents refused to participate, the teams substituted it with an adjacent house. Beside estimating parasitological prevalence by RDT (Rapid Diagnostic Tests, 'FalciVax' by Zephyr Biomedicals, India) from one randomly chosen member (age>1 year) of each household, the survey questionnaire was administered in a face-to-face interview to the participant if adult or the household head (or spouse or any knowledgeable member of the household) if children to elicit information. Information was also collected for any febrile illness in the past 15 days among the household members and relevant health-seeking behaviour. Patients diagnosed as having malaria at the time of survey were referred for treatment as per national guideline.

The day-to-day field activities of the teams were fine-tuned by field researchers based in local offices and supervised by the principal author. Whenever necessary, re-interview was done by the supervisors for securing reliable and valid data. To improve the latter, an independent quality control team spot-checked households randomly within three days of the main survey. In cases where inconsistencies were noted, interviewers were accompanied by field supervisors until quality standards were met.

The variables

When individuals living together took meal from a common cooking facility, the entity is defined as a Household (HH). The head of Household is defined as the person who was perceived by household members to be the primary decision-maker in the family and who may or may not have been the main income-earner. Education was measured by completed years of formal schooling. Engagement in a particular income-earning activity for the major part of the day was categorized as 'main occupation'.

Wealth index was constructed following the method developed by Filmer and Pritchett [13]. The assets included for developing the index were: table, bed-cot, quilt, watch, radio, television, bi-cycle and electricity. Each of the variables was recorded into categorical dichotomous (yes, no) variable. Eight dichotomous variables were created and standardized. The principal component analysis was run with all constructed variables with certain criteria. The component score coefficient matrix was multiplied by the standardized variables to produce factor scores which were termed as household wealth score. The wealth scores were further classified into five quintiles, starting from the lowest (1st quintile, poorest) to highest (5th quintile, least poor).

Ethical considerations

The study received approval by the research review board and the ethical review board of ICDDR, B. Informed consent was obtained from the participants or their guardians before proceeding with the survey activities. Anonymity of the respondents at all stages of data analysis.

Results

Overall malaria prevalence rate in the 13 endemic districts was found to be 3.1% (*Plasmodium falciparum* 2.73%, *Plasmodium vivax* 0.16% and mixed infection 0.19%) by the Rapid Diagnostic Test 'FalciVax' (Zephyr Biomedicals, India) and is reported in detail elsewhere [11]. The prevalence was higher in the five south-eastern districts (7.2% as opposed to 0.5% in the north-eastern districts), with highest concentration in the three hill districts (11%).

The socio demographic and household characteristics of the study population are presented in Table 1 by the two study areas. Majority of the respondents (45%) were in their middle age (40–59 years). A greater proportion of respondents from NE districts (55%) compared to SE districts (45%) did not have any formal schooling, with a sex divide disfavouring the females. For males, involvement in farm activities was the most frequent occupation (about 41%), while this was household chores in case of females (around 65%). Less than 7% of the respondents were from female-headed households. Findings revealed

Table 1: Socio-demographic and household characteristics of the respondents study areas and sex (%)

	South-eastern districts			North-eastern districts		
	M	F	All	M	F	All
Sociodemographic characteristics						
Age (yrs)						
≤19	0.1	0.8	0.2	0.5	0.2	0.5
20–39	38.9	25.4	38.0	35.5	24.8	34.8
40–59	43.7	53.2	44.4	45.3	54.9	45.9
≥60	17.2	20.6	17.5	18.8	20.1	18.9
Completed years of schooling						
None	42.8	69.4	44.6	53.6	75.2	55.1
1–5	25.7	17.9	25.2	24.9	17.6	24.4
>5	31.6	12.7	30.3	21.5	7.1	20.6
Main occupation						
Self-employment (agri.)	45.8	11.1	43.4	42.7	2.9	40.0
Self-employment (non-agri)	23.0	3.2	21.6	21.9	2.9	20.6
Wage-labour	10.2	9.9	10.2	17.0	8.8	16.5
Service	10.6	4.8	10.2	7.4	2.9	7.1
Domestic chores	0.7	61.5	4.8	1.1	69.9	5.8
Others*	9.7	9.5	9.7	9.8	12.5	10.0
Household head						
Male	---	---	93.3	---	---	93.2
Female	---	---	6.7	---	---	6.8
Household asset quintiles						
Poorest	---	---	23.6	---	---	18.0
2nd	---	---	21.3	---	---	21.6
3rd	---	---	18.1	---	---	18.5
4th	---	---	19.7	---	---	20.3
Least poor	---	---	17.4	---	---	21.6
N	3498	252	3750	5548	408	5999

*beggar, unemployed, too old/sick to work etc.

that the households from SE area fared better than the other area when stratified in terms of asset quintiles e.g., the proportion of poorest households was 24% in the SE area compared to 18% in the NE area.

Tables 2, 3 and 4 present malarial awareness of the respondents with respect to sex, educational attainment and household economic status (as proxied by asset quintiles) respectively. In general, the respondents were aware about the cause ('mosquito bite') and presenting symptoms of malarial illness ('fever with shivering') irrespective of sex, and this awareness increased uniformly with years of schooling as well as level of affluence ($p < 0.001$). However, when they were asked about its mode of transmission, only around 39% in the SE area and 32% in the NE area could respond correctly ('by bite of mosquito which has bitten a malarial patient') ($p < 0.001$, Table 2), and was also found to be a factor of schooling years and affluence ($p < 0.001$, Tables 3 and 4 respectively).

Interestingly, the use of bed net for prevention of malaria was singled out uniformly by the respondents (>80%,) (Table 2). Other measures reported were: preventing breeding of mosquito (13% in SE area and 18% in NE area, $p < 0.001$), using mosquito repellant/coil (16% in SE area and 20% in NE area, $p < 0.001$). Though there was not much variation by sex (Table 2), but the trend observed earlier with education and affluence remained valid with one exception (Tables 3 and 4 respectively). The respondents almost unanimously reported allopathic medicine to be the treatment for malaria (>98%), especially the more educated and the affluent ones (Tables 3, 4 respectively).

Neighbours and relatives were the most frequently mentioned group for malaria-related information (35% in SE and 49% in NE areas respectively, $p < 0.001$) by the respondents, especially the females (Table 2). However, with increasing level of education and affluence ($p < 0.001$, Tables 3 and 4 respectively), the proportion

Table 2: Reported knowledge on malaria by study areas and sex (multiple responses)

	South-eastern districts			North-eastern districts			χ^2 significance
	M	F	All	M	F	All	
	a	b	c	d	e	f	c vs f
Causes of malaria							
Mosquito bite	90.7	89.2	90.6	94.4	91.9	94.2	p < 0.001
Fly/insect bite	4.8	4.0	4.7	2.0	1.7	2.0	p < 0.001
Not maintaining neat and cleanliness	10.5	7.6	10.3	16.0	5.2	15.9	p < 0.001
Others	3.5	4.2	5.4	3.5	4.2	3.6	p < 0.001
Symptoms of malaria							
Onset of fever with shivering	76.4	72.1	76.1	83.2	81.1	83.0	p < 0.001
Fever at intervals	19.8	22.3	21.0	25.6	27.7	25.8	p < 0.001
Remission of fever with sweating	10.5	7.6	10.3	16.0	15.2	15.9	p < 0.001
Others	18.5	18.7	18.5	10.3	8.6	10.2	p < 0.001
Mode of transmission							
By bite of any mosquito	33.4	36.3	33.5	32.9	36.0	33.1	ns
By bite of mosquito which has bitten a malarial patient	39.2	29.9	38.6	32.5	31.9	32.4	p < 0.001
Don't know	24.4	28.3	24.7	30.6	27.7	30.4	p < 0.001
Other	3.1	3.6	3.1	6.9	7.4	6.9	p < 0.001
Mode of prevention							
Preventing breeding of mossquito	13.3	6.8	12.9	17.6	18.6	17.7	p < 0.001
Using bednet	84.1	83.3	84.0	84.3	80.9	84.1	ns
Using insecticide impregnated bednet	1.0	0.8	1.0	2.1	2.7	2.1	p < 0.001
Using mosquito repellent/coil	16.1	13.1	15.9	19.9	20.8	20.0	p < 0.001
Other	12.3	15.1	12.5	9.4	10.5	9.5	p < 0.001
Mode of treatment							
Allopathic treatment	96.0	96.0	96.0	98.3	97.3	98.2	p < 0.001
Traditional (Herbal/Kabiraji)	1.7	1.2	1.7	1.1	2.0	1.1	p < 0.01
Faith healing	0.5	0.0	0.3	0.3	0.2	0.3	ns
Homeopathic	0.5	0.0	0.3	0.3	0.2	0.3	ns
Other	0.8	1.2	0.8	0.2	0.5	0.2	p < 0.001
Source of information							
Govt. health worker	21.2	23.5	20.7	25.1	23.5	25.0	p < 0.001
NGO health worker	26.8	18.7	26.3	16.7	14.2	16.5	p < 0.001
Radio/TV/Newspaper	14.5	15.5	14.6	15.2	13.0	15.0	ns
Poster/leaflet	2.4	5.6	2.6	12.9	3.2	2.0	p < 0.05
Neighbours/relatives	34.8	43.8	35.4	48.2	27.1	48.8	p < 0.001
Self	16.9	13.9	15.8	14.7	9.6	11.4	p < 0.05
Other	5.7	6.0	5.7	4.2	4.2	4.2	p < 0.001
N	3499	251	3750	5591	408	5999	

decreased gradually to be replaced by community health workers from government and NGOs. Mass media (Radio/TV/Newspaper) and printing media (poster/leaflet) became increasingly important means of message dissemination in those instances.

Around 2% of the respondents in SE area and 0.4% respondents in NE area reported to have had suffered from fever with shivering within 15 days prior to the day of survey ($p < 0.001$). No sex difference in fever prevalence was seen (Table 5). Next, information on their health-seeking behaviour was elicited. Majority of these patients did not seek any treatment, women more so than men

Table 3: Reported knowledge on malaria by study areas and completed years of schooling (multiple responses)

	South-eastern districts				North-eastern districts			
	Completed years of schooling							
	None	1–5	>5	χ^2 significance	None	1–5	>5	χ^2 significance
Causes of malaria								
Mosquito bite	87.4	91.6	94.7	p < 0.001	93.0	95.0	96.6	p < 0.001
Fly/insect bite	4.5	4.5	5.2	ns	1.6	1.9	3.1	p < 0.01
Not maintaining neat and cleanliness	8.3	11.6	12.1	p < 0.001	15.0	13.6	21.2	p < 0.001
Others	7.1	4.5	3.7	p < 0.001	4.4	3.0	1.9	p < 0.001
Symptoms of malaria								
Onset of fever with shivering	71.9	77.2	81.6	p < 0.001	80.0	84.9	88.8	p < 0.001
Fever at intervals	19.2	18.4	22.5	p < 0.05	26.8	22.7	26.7	p < 0.05
Remission of fever with sweating	8.8	11.6	12.1	p < 0.001	15.0	13.6	21.2	p < 0.001
Others	18.5	17.6	19.2	ns	10.9	9.4	9.2	ns
Mode of transmission								
By bite of any mosquito	34.9	37.2	28.4	p < 0.001	33.2	35.7	30.0	p < 0.05
By bite of mosquito which has bitten a malarial patient	29.2	38.9	52.2	p < 0.001	27.5	28.5	49.8	p < 0.001
Don't know	29.2	21.2	20.8	p < 0.001	33.6	31.9	20.2	p < 0.001
Other	3.2	3.1	3.0	ns	7.7	6.3	5.6	p < 0.05
Mode of prevention								
Preventing breeding of mosquito	7.8	12.9	20.3	p < 0.001	16.8	14.0	24.4	p < 0.001
Using bed net	80.9	84.4	88.4	p < 0.001	81.4	85.2	89.7	p < 0.001
Using insecticidal bed net	0.8	1.1	1.3	ns	1.6	2.6	2.9	p < 0.01
Using mosquito repellent/coil	11.2	17.1	22.1	p < 0.001	15.6	21.6	30.0	p < 0.001
Other	14.2	12.4	10.2	p < 0.05	10.9	9.0	6.5	p < 0.001
Mode of treatment								
Allopathic treatment	94.3	96.2	98.3	p < 0.001	97.8	98.3	99.0	p < 0.02
Traditional (Herbal/Kabiraji)	2.0	1.4	1.4	ns	1.0	1.4	1.1	ns
Faith healing	0.5	0.5	0.5	ns	0.2	0.3	0.3	ns
Homeopathic	0.5	0.4	0.3	ns	0.2	0.3	0.6	ns
Other	1.2	0.7	0.4	p < 0.05	0.3	0.1	0.0	ns
Source of information								
Govt. health worker	17.8	19.9	25.5	p < 0.001	22.7	23.5	32.9	p < 0.001
NGO health worker	23.6	25.9	30.6	p < 0.001	15.0	17.0	20.5	p < 0.001
Radio/TV/Newspaper	9.4	16.5	20.7	p < 0.001	12.0	13.4	24.8	p < 0.001
Poster/leaflet	1.5	2.8	4.2	p < 0.001	2.0	1.1	2.9	p < 0.01
Neighbours/relatives	41.6	35.7	25.8	p < 0.001	54.7	48.5	33.2	p < 0.001
Self	13.3	16.6	19.5	p < 0.001	12.0	16.5	18.0	p < 0.001
Other	6.5	6.5	4.0	p < 0.01	4.5	4.5	3.3	ns
N	1683	938	1129		3322	1453	1224	

and those from SE area more so than those in the NE area. Self-treatment was practiced more frequently by patients from NE area (14%) than by those from SE area (11%). Professional allopathic practitioners were consulted in 13% of instances in both areas, with a gender gradient disfavoring women. On the other hand, drugstore salespeople were consulted more frequently by those from the SE area (47%) compared to the NE area (32%), with marginal or no gender difference (Table 5). When disaggregated by education and SES, a decrease in proportion of no-treatment and a simultaneous increase in treatment seeking from professional allopaths i.e., MBBS doctors was observed with increasing level of education and affluence (Table 6).

Finally, Table 7 shows that there was delay in initiation of treatment beyond 24 hours in majority of the instances, and the illness also prolonged beyond seven days, especially in the NE area ($p < 0.01$). This resulted in the disruption of income-earning activities beyond five days, more so in the NE area ($p < 0.01$).

Discussion

The role of social science research in the design and implementation of evidence-based prevention, management

Table 4: Reported knowledge on malaria by study areas and wealth quintiles (multiple responses)

	South-eastern districts				North-eastern districts			
	Household asset quintiles							
	Poorest	3rd Quin.	Least poor	χ^2 significance	Poorest	3rd Quin.	Least poor	χ^2 significance
Causes of malaria								
Mosquito bite	84.8	90.7	95.5	p < 0.001	92.2	93.2	96.7	p < 0.001
Fly/insect bite	4.6	5.0	6.5	ns	0.8	1.6	4.4	p < 0.001
Not maintaining neat and cleanliness	7.9	10.6	13.4	p < 0.01	10.1	16.6	22.6	p < 0.001
Others	8.0	5.3	3.4	p < 0.001	5.0	4.3	1.6	p < 0.001
Symptoms of malaria								
Onset of fever with shivering	68.7	76.8	82.9	p < 0.001	76.3	85.0	86.5	p < 0.001
Fever at intervals	17.6	20.6	21.0	ns	25.7	21.7	32.1	p < 0.05
Remission of fever with sweating	7.9	10.6	13.4	p < 0.01	10.1	16.6	22.6	p < 0.001
Others	21.9	17.8	16.1	p < 0.01	12.7	9.0	9.8	p < 0.001
Mode of transmission								
By bite of any mosquito	30.8	33.4	39.3	p < 0.05	29.9	30.1	38.6	p < 0.001
By bite of mosquito which has bitten a malarial patient	30.5	40.1	43.0	p < 0.001	23.7	30.9	43.7	p < 0.001
Don't know	31.0	32.9	18.6	p < 0.001	39.5	32.0	19.3	p < 0.001
Other	1.9	4.0	3.7	p < 0.05	6.4	9.7	4.9	p < 0.001
Mode of prevention								
Preventing breeding of mosquito	6.8	13.1	17.8	P < 0.001	8.9	17.9	26.4	p < 0.001
Using bed net	70.1	84.4	89.4	P < 0.001	84.6	80.4	87.1	p < 0.001
Using insecticidal bed net	0.7	1.5	1.2	ns	0.9	2.7	2.3	p < 0.05
Using mosquito repellent/coil	8.0	15.6	24.6	P < 0.001	13.2	16.4	32.6	p < 0.001
Other	16.9	12.5	8.8	p < 0.001	10.1	11.9	7.0	p < 0.001
Mode of treatment								
Allopathic treatment	92.6	95.7	98.6	p < 0.001	97.2	98.5	98.6	p < 0.05
Traditional (Herbal/Kabiraji)	2.4	1.6	1.7	ns	1.2	1.0	1.2	ns
Faith healing	0.2	0.6	0.6	ns	0.2	0.2	0.4	ns
Homeopathic	0.2	0.1	0.5	ns	0.1	0.2	0.5	ns
Other	1.5	0.6	0.0	p < 0.05	0.6	0.0	0.0	p < 0.001
Source of information								
Govt. health worker	17.8	21.6	22.1	ns	28.1	22.3	26.2	p < 0.05
NGO health worker	23.9	29.7	19.5	p < 0.001	14.3	17.5	16.7	ns
Radio/TV/Newspaper	5.3	13.5	32.4	p < 0.001	4.1	10.5	31.6	p < 0.001
Poster/leaflet	0.9	1.8	6.9	p < 0.001	0.3	2.1	2.7	p < 0.001
Neighbours/relatives	43.9	35.0	28.1	p < 0.001	50.6	53.7	37.9	p < 0.001
Self	12.4	15.0	21.4	p < 0.001	13.3	13.8	18.3	p < 0.001
Other	5.2	6.8	5.4	ns	6.5	3.8	3.6	p < 0.001
N	884	680	651		1082	1108	1294	

and control strategies for malaria cannot be overemphasized [14]. There is lack of this kind of data in Bangladesh and this baseline survey on malaria has attempted to fill in this knowledge gap besides estimation of parasitological prevalence from a population based survey in the 13 endemic districts. Findings revealed superficial knowledge on malarial transmission, prevention and treatment, especially among the poor and the illiterate. A gender and geographical divide with respect to different aspects of malaria prevention and treatment was observed, disfavouring women and south-eastern area respectively. While the respondents preferred allopathic providers for treatment of malaria unanimously, a 'know-do' gap in practices existed. In about half of the instances, a delay in seeking care for malaria-like fever was observed. These are discussed below with its implications for programme implementation

The awareness of the respondents that malaria is caused and transmitted by bite of mosquito is usually a common

Table 5: Prevalence of fever with shivering in past 15 days prior to the day of survey and relevant health-seeking behaviour %

	South-eastern districts			North-eastern districts			χ^2 significance
	M	F	All	M	F	All	
	a	b	c	d	e	f	C vs f
Had fever with shivering in last 15 days	1.7	2.0	1.8	0.5	0.4	0.4	$p < 0.001$
N	10147	9675	19822	16025	14962	30987	
Health-seeking behaviour							
No treatment	35.8	41.3	38.7	20.5	25.4	22.7	
Self-treatment	10.4	11.1	10.8	15.1	11.9	13.6	
Drug store salespeople	35.3	29.6	32.3	45.2	49.2	47.0	
Paraprofessionals	2.3	4.8	3.6	2.7	3.4	3.0	
Professional allopaths (MBBS doctors)	14.5	11.6	13.0	16.4	8.5	12.9	
Others	1.7	1.6	1.7	0.0	1.7	0.8	
χ^2 significance	ns			ns			
N	173	189	362	73	59	132	

knowledge in malaria endemic countries such as India, Turkey, Nepal, Haiti, Latin America, Sudan and Ghana [15-22]. However, only a tiny fraction of the respondents could accurately state the correct transmission route ('by bite of mosquito which has bitten a malarial patient') and none could state how the mosquito becomes infective i.e., the parasitological cause. The serious gaps in knowledge are also revealed by one-third of the respondents stating that they did not knew the mode of transmission and another one-third stating that any mosquito bite causes malaria. The poor and the semi-literate are especially disadvantaged in these aspects. Health education interventions should be designed according to the existing knowledge and awareness level of vulnerable population as well as their current treatment-seeking practices, and should be implemented for sufficient length of time to be effective [20].

The association of febrile illness with malaria has been known in Bangladesh for a long time [23]. This is also reiterated in this study, where the majority of the respondents mentioned fever (with shivering, at intervals) as the most common symptom of malaria and is consistent with observations from other countries [16,18,20,21,24-26].

Knowledge on the use of bed net as a preventive measure against mosquito bite was high among the respondents in this study. Similar high level of knowledge on preventive use of bed net had been observed in Nepal [18] and Ghana [22], but not in countries such as Ethiopia [25], Iran [26], Delhi, India [16], Turkey [17], and Haiti [19]. This advantage will make the work of the programme easier in introducing insecticidal bed nets (LLINs/ITNs) as a strategic measure for preventing malaria transmission. However, the programme also needs to keep the equity perspective in focus, while distributing insecticidal bed nets, because the poorer households were found to be disadvantaged in this respect.

The respondents were unanimous about seeking treatment from the allopathic providers, whether in the formal or informal sector. However, the 'know-do' gap became especially evident when in practice majority of the ill persons either did not seek any treatment or practiced self-treatment. The latter is consistent with findings from Turkey, where the majority practice self-treatment for malaria [17]. Of those who sought treatment, the majority went to the informal allopathic providers, such as village doctors and drugstore salespeople whose knowledge and capacity for curative treatment is not without question [27]. Also, there was a delay in the beginning of treatment in more than half of the instances of febrile episodes suggestive of malaria, and there was disruption of income-earning activities due to prolonging of the illness. Thus, efforts will be needed to educate this population on the need for 'Early Diagnosis and Prompt Treatment (EDPT)' for reducing its income-erosion effect. Further, the capacity of the informal allopathic providers (important for treatment of poor) should be developed in the use of Rapid Diagnostic Tests (RDTs), and the rational use of artemisinin-based combination drugs (such as Coartem®) so as to fast-track informed diagnosis and treatment.

Table 6: Health-seeking behaviour of patients by education and affluence %

	South-eastern districts			North-eastern districts		
	Years of schooling					
	None	1–5	>5	None	1–5	>5
No treatment	39.1	41.1	28.8	20.7	26.3	10.0
Self-treatment	12.8	7.5	15.3	10.3	13.2	25.0
Drug store salespeople	33.8	31.8	32.2	55.2	39.5	35.0
Paraprofessionals	4.5	1.9	5.1	0.0	5.3	10.0
Professional allopaths (MBBS doctors)	9.0	15.9	16.9	12.1	15.8	20.0
Others	0.8	1.9	1.7	1.7	0.0	0.0
χ^2 significance	ns			ns		
N	133	107	59	58	38	20
	Household asset quintiles					
	South-eastern districts			North-eastern districts		
	Poorest	3rd Quin.	Least poor	Poorest	3rd Quin.	Least poor
No treatment	47.2	30.6	27.6	29.2	28.6	16.0
Self-treatment	10.4	14.5	10.3	16.7	14.3	24.0
Drug store salespeople	26.4	30.6	37.9	37.5	52.4	36.0
Paraprofessionals	3.2	6.5	3.4	0.0	0.0	0.0
Professional allopaths (MBBS doctors)	11.2	12.9	17.2	12.5	4.8	24.0
Others	1.6	4.8	3.4	4.2	0.0	0.0
χ^2 significance	ns			ns		
N	125	62	29	24	21	25

Throughout this study gender divide in knowledge, awareness and health-seeking behaviour was observed disfavouring women. This is not surprising, given the patriarchal norms in the society and was also noted earlier in other studies [28]. While designing interventions, proactive measures should be undertaken by malarial prevention and control programmes to reduce this gender gap. This is all the more necessary because experiences show that even women-focused interventions may not increase access of quality health care for women, if the gender issues are not explicitly addressed by the programme [29].

Lastly, there are regional differences. The SE area was found to have greater proportion of poorest households (in terms of asset quintiles) than the NE area. The SE area respondents also appeared to be disadvantaged regarding different aspects of malaria prevention and treatment than the NE area, though marginally. However, this difference has to be taken into consideration while allocating resources for specific interventions.

Conclusion

The findings of the survey have important implications for fine-tuning the current malaria prevention and control programme. The programme should disseminate comprehensive information on different aspects of malaria for converting the 'unfelt' need to 'felt' need of the community to facilitate the uptake of preventive and curative measures. This is all the more necessary as it has been found elsewhere that knowledge of malaria influences the use of preventive measures such as use of insecticidal nets (30). Intensive campaign for practicing EDPT is necessary so that the community is convinced about its need for reducing malaria mortality, especially among the vulnerable groups. Besides print and electronic media, various informal communication methods (e.g., folk songs, people's theatre etc.) can be used to reach the disadvantaged sections of this largely illiterate community. Finally, equity focus in terms of gender, SES and geographical location should be maintained at every stage of programme implementation.

Table 7: Time to treatment initiation, duration, disruption of income-earning and illness expenditure by study areas and sex

	South-eastern districts			North-eastern districts			significance
	M	F	All	M	F	All	
	a	b	c	d	e	f	c vs. f
Treatment initiated %							p < 0.01
Within 24 hours	44.8	41.7	43.2	24.6	27.3	25.7	(χ^2)
Beyond 24 hours	55.2	58.3	56.8	75.4	72.7	74.3	
Duration of illness %							p < 0.01
≤ 3 days	29.1	33.9	31.7	19.6	21.9	20.5	(χ^2)
4–7 days	50.5	36.4	43.0	28.3	37.5	32.1	
≥ 7 days	20.4	29.7	25.3	52.2	40.6	47.4	
Days income-earning was disrupted (mean)	5.0	5.3	5.1	7.0	9.2	7.6	p < 0.003 (t-test)

*beggar, unemployed, too old/sick to work etc.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SMA conceptualized and designed the study, analysed data, drafted the manuscript and made final revisions. RH did sample calculations and designed the study, analysed data and made critical revision of the manuscript. UH organized the field activities, analysed data and helped in the revision of the manuscript. AH analysed data, made the tables and helped in drafting the manuscript. All authors read the final manuscript and approved.

Additional material

Additional file 1

***Malaria Baseline Survey 2007: Questionnaire**. Questionnaire used in the Baseline Survey.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1475-2875-8-173-S1.pdf]

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Spectrum of complications associated with *Plasmodium vivax* infection in a tertiary hospital in South-Western India

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ABSTRACT

Objective: To determine the range and incidence of complications associated with *Plasmodium vivax* (*P. vivax*) malaria. **Methods:** A retrospective analysis was performed of all patients of *P. vivax* malaria admitted in Kasturba Medical College, Manipal between January and December, 2010. Patients with mixed malarial infection were excluded by appropriate tests. Clinical presentation and laboratory parameters were studied. **Results:** Medical records of 213 individuals who satisfied the inclusion criteria were reviewed. Anaemia was seen in 65 (30.5%), leucopenia in 38 (17.8%) and thrombocytopenia in 184 (86.4%) patients. Aspartate and alanine aminotransferases were elevated in 86 (40.4%), and 89 (41.9%) patients respectively. Hypoalbuminemia was observed in 157 (73.6%) cases. Elevated serum creatinine was noted in in 59 (27.5%) patients. Creatine kinase was elevated in 30 out of 59 patients (50.8%). Overall, 107 (50.2%) patients fulfilled WHO criteria for sever=e malaria. None of the patients succumbed to the disease. **Conclusion:** *P. vivax* malaria is a potentially severe disease, and the term "benign" tertian malaria is a misnomer. Despite significant morbidity, with timely and appropriate treatment *P. vivax* malaria has an excellent outcome.

1. Introduction

Plasmodium vivax (*P. vivax*) malaria, also known as benign tertian malaria, is a tropical disease with a global distribution. Once a dreaded infection, *P. vivax* malaria has now lost much of its notoriety, principally due to the widespread availability of powerful and efficacious antimalarial drugs. Moreover, the relatively greater severity of infection with *Plasmodium falciparum* (*P. falciparum*) has resulted in a shift of international policy and resources towards this more virulent form of malaria. While it is certainly true that *P. falciparum* is a source of significant morbidity and mortality, it is pertinent to note that a large fraction of the malarial burden outside of Sub-Saharan Africa remains attributable to *P. vivax*. The sheer burden of disease is astounding with annual global incidence estimated at 225 million[1]. The incidence in India is estimated at 1.5 million cases annually of which nearly 40% are due to *P. vivax*. In light of these statistics, we designed this study to focus on the various clinical and biochemical abnormalities associated with *P. vivax* malaria.

2. Materials and methods

2.1. Study settings

Kasturba Hospital, Manipal is a major tertiary care hospital in Southwestern India. For the purpose of this study, the catchment area of this hospital corresponds to the district of Udupi in which it is situated as well as the neighbouring districts of Uttara Kannada and Dakshina Kannada, with a combined population of approximately 4.36 million individuals.

A retrospective analysis of data of patients with *P. vivax* malaria admitted between January and December 2010 was conducted from medical records. All cases with mixed malarial infection *i.e.* both *P. vivax* and *P. falciparum* were excluded by means of peripheral blood smear examination and serological testing for histidine rich protein 2 by the Falcivax test (Zephyr Biomedical Systems, Goa, India). All patients included in the study had tested positive for *P. vivax* malarial parasite by either peripheral smear examination with Giemsa staining or fluorescein staining of buffy coat layer. Findings of clinical examination were recorded. Laboratory parameters including complete blood counts, renal and liver function tests, creatine kinase

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and blood glucose were collected. WHO criteria for severe malaria were used to identify patients with severe *P. vivax* malaria.

Primary outcome was defined as mortality due to infection with *P. vivax* malaria or complications thereof. Secondary outcomes included the various complications developed.

2.2. Statistical analysis

All data was analysed using SPSS Statistics version 17.0 (Chicago IL, USA). Continuous variables were presented as mean ± standard deviation (SD).

3. Results

213 patients who fulfilled the inclusion criteria were included in the study. Of them, 172 (80.8%) were males and 41 (19.2%) were females. Mean age was (33.17±14.85) years. Cases of malaria were seen throughout the year peaking between the months of May and August, corresponding to the Monsoon season (Figure 1).

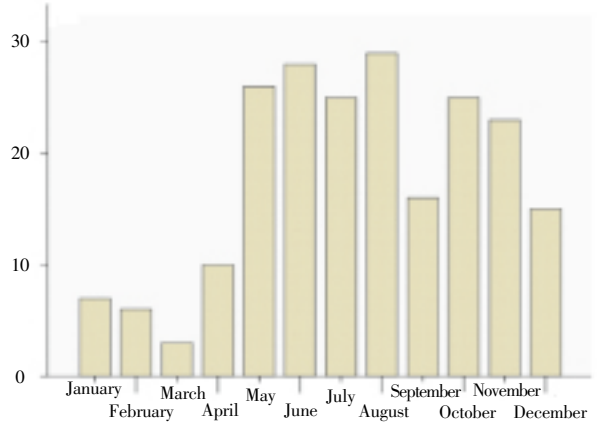

Figure 1. Monthly incidence of *P. vivax* malaria.

The principal presenting complaint was fever, which was present in all the cases. Two patients presented with hyperpyrexia. Seizures and altered sensorium were observed in three patients. Clinical examination showed hypotension in ten patients. Chest radiograms were suggestive of acute respiratory distress syndrome (ARDS) in four patients.

Analysis of complete blood counts revealed anaemia in 65 (30.5%) patients, leucopenia in 38 (17.8%) patients and thrombocytopenia in 184 (86.4%) patients. Liver function tests were also deranged; indirect hyperbilirubinemia was seen in 85 (39.91%) patients, direct hyperbilirubinemia was seen in 26 (12.21%). Malarial hepatitis was also observed, 86 (40.4%) patients had elevated serum aspartate aminotransferase levels, while 89 (41.9%) had elevated levels of serum alanine aminotransferase. Elevated alkaline phosphatase levels were noted in 40 (19%) patients. Hypoalbuminemia was seen in 157 (73.6%) patients while hypergammaglobulinemia was noted in 21 (10.1%) patients. Renal failure was encountered frequently; 44 (20.7%) patients had elevated blood urea levels, while serum creatinine was high in 59 (27.5%) patients. 27 (13%) patients had hypokalemia. 30 out of 59 (50.8%) patients had elevated serum creatine kinase levels.

These findings are summarized in Table 1. Patients were also scored according to the WHO criteria for severe malaria[2]. Neurological involvement in the form of seizures and coma were encountered in 3 (1.41%) patients, while severe anaemia (Hb < 5 g/dL) and renal failure (creatinine > 3 mg/dL) were seen in 1 (0.47%) and 2 (0.94%) patients respectively. Adult respiratory distress syndrome (ARDS) and shock were noted in 4 (1.88%) and 10 (4.69%) patients. Jaundice (serum bilirubin > 3.0 mg/dL), elevated liver enzymes (AST/ALT > 3 times upper limit of normal) and thrombocytopenia ($< 5\times10^4/mm^3$) were observed in 29 (13.62%), 13 (6.10%) and 68 (31.92%) patients respectively. Elevated muscle enzymes were noted in 30 of 59 (50.8%) patients in whom it was estimated. Hypoglycemia (Plasma glucose < 40 mg/dL), and disseminated intravascular coagulation were not encountered in any patients.

Overall, 107 (50.2%) patients were found to have severe malaria. Despite the frequency of complications observed, all patients included in the study survived. The primary outcome of mortality was therefore not seen in this study.

Table 1
Laboratory abnormalities encountered.

Parameter	Patients with deranged parameter (%)	Mean±SD
Haemoglobin	65 (30.50%)	12.92±2.24
Total leucocyte count	38 (17.80%)	6 019.25±2 493.17
Platelet count	184 (86.40%)	84 053.40±59 255.64
Indirect bilirubin	85 (39.91%)	–
Direct bilirubin	26 (12.21%)	1.15±2.61
AST[a]	86 (40.40%)	47.06±48.42
ALT[b]	89 (41.90%)	46.69±43.23
ALP[c]	40 (19.00%)	103.38±59.10
Albumin	157 (73.60%)	3.53±0.68
Globulin	21 (10.10%)	2.97±0.60
Blood urea	44 (20.70%)	34.68±23.94
Creatinine	59 (27.50%)	1.14±0.46
Potassium	27 (13.00%)	3.94±0.51
Creatine kinase	30/59 (50.80%)	196.44±247.593

a: AST=Aspartate aminotransferase; b: ALT=Alanine aminotransferase; c: ALP=Alkaline phosphatase.

4. Discussion

Although less virulent than *P. falciparum*, *P. vivax* can nevertheless produce severe complications[3]. The frequency of such complications as observed in our study is alarming, especially in the context of the massive global burden of *P. vivax* malaria. Within India, the estimated annual incidence of malaria is around 1.5 million cases, of which over 40% are due to *P. vivax*[1]. Moreover, over 80% of the Indian population is estimated to be at risk of infection with malaria.

In our study, thrombocytopenia was the commonest complication, seen in 184 (86.4%) patients. Severe thrombocytopenia below $20\times10^3/mm^3$ was observed in 17 (8.3%) patients; however, none of these patients displayed bleeding tendencies. In their study on *vivax* malaria, Sharma and Khanduri[4] reported thrombocytopenia in 96.4% patients with severe thrombocytopenia in 6% patients. Kochar *et al*[5] detected thrombocytopenia in 22.5% patients. Cases of severe thrombocytopenia have also been reported by Makkar *et al*[6] and Rodriguez *et al*[7]. Postulated mechanisms for thrombocytopenia include macrophage activation, increased levels of cytokines and antiplatelet immunoglobulin resulting in accelerated destruction of platelets. Other putative mechanisms include oxidative stress, sequestration in non-splenic areas and pseudothrombocytopenia due to clumping of platelets[8].

Deranged liver function tests were frequently noted in our study. 29 (13.62%) patients had serum bilirubin levels greater than 3 mg/dL and 13 (6.10%) had serum aminotransferases elevated over three times the upper limit of normal. Although, Sharma and Khanduri[4] detected these abnormalities in only 4% and 3.17% of patients respectively, they found higher levels of serum aminotransferases in individual patients, with aspartate aminotransferase and alanine aminotransferase values upto 1 901 U/L and 848 U/L respectively. Corresponding values noted in our study were 511 U/L and 434 U/L. In contrast, Kochar *et al*[5] reported hepatic dysfunction in 57.5% of their patients. Such cases of 'malarial hepatitis' are more typical of *P. falciparum*[9], but have occasionally been reported with *P. vivax*[10].

Renal failure was observed in 59 (27.5%) of our patients, although elevation of serum creatinine beyond 3 mg/dL was noted in only 2 (0.94%) patients. The highest level of serum creatinine seen was 3.9 mg/dL. *P. vivax* malaria has been reported in a number of studies[11-15] to be responsible for between 2.42% and 12.5% of all cases of malarial renal failure. Sharma and Khanduri[4] detected renal failure in 7% of their cases while Kochar *et al*[5] found renal failure in 45% of their patients.

Interestingly, the paediatric age group appears to be at a greater risk for renal failure following infection with *P. vivax*. Kaur *et al*[16] reported a paediatric case with uremic encephalopathy secondary to *P. vivax* induced renal failure. Haemolysis, volume depletion, disseminated intravascular coagulation, rhabdomyolysis[17], hyperbilirubinemia and heavy parasitemia[18] are among the various factors implicated in the pathogenesis of renal failure in malaria. Cytoadherence has also been suggested as a mechanism although its role appears to be only minimal[19].

Acute respiratory distress syndrome (ARDS) was seen in 4 (1.88%) patients in our study. Sharma and Khanduri[4] reported three cases of ARDS out of 221 patients with *P. vivax* malaria, while Kochar *et al*[5] noted ARDS in four cases out of 40 patients. Suggested mechanisms include small airway obstruction, impaired gas exchange, increased phagocytic activity and accumulation of pulmonary mononuclear cells[20]. Intriguingly, these underlying pathophysiologic processes appear to be independent of parasite sequestration within the pulmonary vasculature[21]. This point is borne out by the fact that patients with *P. vivax* malaria often develop ARDS after completing antimalarial therapy[22], suggesting an inflammatory rather than an infectious basis for this complication. In this context, the use of corticosteroid therapy in malarial ARDS remains controversial[23] although it has been used with some success in the treatment of another rare pulmonary complication of malaria-bronchiolitis obliterans organizing pneumonia.

Neurological involvement in the form of cerebral malaria is commonly encountered in *P. falciparum* malaria, but extremely unusual with *P. vivax*. Indeed only 42 cases have been reported in medical literature[24]. Kochar *et al*[5] diagnosed cerebral malaria in five cases. In our study, we found three patients with symptoms fitting into cerebral malaria including recurrent seizures in two cases and persistent confusion in one case. Numerous pathologic processes for cerebral malaria have been proposed including adherence of parasitized red cells to the cerebral vascular endothelium, fibrin microthrombi, agglutination of parasitized red cells and dysregulated local nitric oxide production. While *P. vivax* has traditionally been considered to be incapable of inducing cytoadherence, newer evidence suggests that such a phenomenon is indeed possible[25,26]. Similar to malarial renal failure, cerebral malaria with *P. vivax* occurs more often in the pediatric age group. Our patients were aged 14, 22 and 35 years. An important possibility to be ruled out in all such cases is that of mixed infection with *P. vivax* and *P. falciparum*. In all our three cases, tests for *P. falciparum* including both peripheral blood smear examination and serologic testing for falciparum antigen were negative, making such a possibility extremely unlikely[24].

Elevated serum creatine kinase levels are typical of *P. falciparum*[17], often in association with acute renal failure. Unexpectedly, elevated serum creatine kinase was seen in 30 out of 59 patients in whom it was done, including all the three cases of cerebral malaria, presumably as a result of seizures. The mean serum creatine kinase in these patients was (196.440±247.593) U/L, with a maximum level of 1 099 U/L; creatine kinase over 1 000 U/L was seen in two patients, both of whom had seizures. However, none of these patients developed classical rhabdomyolysis, as evidenced by myoglobinuria. While occasionally associated with *P. falciparum*, rhabdomyolysis is rare in *P. vivax* malaria. Siqueira *et al*[27] reported a case of *P. vivax* malaria with rhabdomyolysis; Poels *et al*[28] described rhabdomyolysis following *P. vivax* malaria in an individual with myoadenylate deaminase deficiency. TNF-α (myotoxin), red-cell sequestration in skeletal muscle, toxins derived from the parasite, and lactic acidosis have all been proposed as triggers for myositis, myonecrosis, and rhabdomyolysis.

Hypotension defined as a systolic blood pressure below 80 mmHg in adults and below 50 mmHg in children 1-5 years, was observed in 10 (4.69%) patients in our study. Kochar *et al*[5] noted hypotension in three cases. Proposed mechanisms for hypotension in malaria include metabolic acidosis, gastrointestinal bleeding, splenic rupture, dehydration and secondary bacterial septicemia. In our study, bacterial sepsis was ruled out by negative blood cultures in all the affected patients. Hypotension often carries a poor prognosis, in part due to pulmonary edema which can develop following rapid infusion of saline[29].

Severe anaemia below 5 g/dL was seen in only one patient; Kochar *et al*[5] also detected this complication in just one case. The relatively low parasite biomass of *P. vivax* indicates the presence of mechanisms beyond mechanical destruction of infected red cells alone. Malariotherapy studies have shown that for every infected erythrocyte destroyed during vivax infection, 32 noninfected erythrocytes are removed from the circulation compared to the loss of eight erythrocytes for every infected erythrocyte in falciparum malaria[30]. Cytokine-related dyserythropoiesis also probably contributes to anaemia[31].

Of note, certain complications known to occur with severe malarial infection were not seen at all in our study. These included hypoglycemia, hyperparasitemia and disseminated intravascular clotting, as evidenced by prolonged clotting times and the presence of schistocytes in peripheral blood smear. In contrast, complications like elevated muscle enzyme levels and hypoalbuminemia were seen frequently. Rare complications of *P. vivax* malaria such as cerebral malaria and ARDS were also encountered. The reason for this pattern of complications is not clear, and needs to be followed up in larger studies.

Of even greater significance was our finding that 107 (50.2%) patients fulfilled established criteria for severe malaria. This figure is especially daunting when factored with the magnitude of *P. vivax* malaria infection worldwide, and underscores the potentially massive morbidity this disease is capable of inflicting, and consequently the need to divert adequate resources towards combating this disease.

In summary, *P. vivax* malaria remains an important source of morbidity, albeit overshadowed by *P. falciparum*. Although infection with *P. vivax* was not associated with mortality, the high frequency of complications observed make the term "benign" tertian malaria a misnomer.

Conflict of interest statement

We declare that we have no conflict of interest.

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The emerging trends of falciparum malaria: a study from a tertiary centre in an endemic area of India

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PEER REVIEW

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Comments

This is a well-written manuscript on Falciparum malaria in an endemic area of India. The results are interesting and suggested that 93 cases had severe malaria high parasite index and abnormal renal function tests are predictors of mortality and complications of disease.
Details on Page S85

ABSTRACT

Objective: To document the various clinical manifestations, lab parameters, complications and outcomes of Falciparum Malaria. The above data would be correlated with the parasitic index to deduce whether it would be an effective measure of the same.
Methods: This was a prospective study among 183 inpatients aged above 18 from Kasturba Hospital, Manipal from May 2009 to January 2011. Ethical clearance was taken. Statistical analysis was done with the independent paired *t* test, linear correlation and *Chi* square test using SPSS 16.
Results: In this study 78% cases were males. Most cases occurred during the monsoons. Fever was the major presentation with others being jaundice, vomiting and head ache. 50.8 % had complications, including hepatic dysfunction (40.9%), renal failure (19.13%), shock (7%), altered sensorium (9%), ARDS (3.27%) and severe anemia (1.63%). Hypoglycemia and gram negative sepsis were rare. Parasitic index, renal parameters and death were correlating positively. ESR was significantly related ($P<0.003$) to complications and not to cerebral malaria. There were 12 mortalities out of which 9 were due to MODS and 3 due to ARDS.
Conclusions: 50.8% cases conformed to the WHO definition of severe malaria indicating most present with complications. High parasite index and abnormal renal function are predictors of mortality and complications. Early diagnosis, anticipation of complications, close monitoring and combination therapy to over come drug resistance helps to contain the extent of mortality.

KEYWORDS

Falciparum malaria, Severe malaria, Parasitic index, Complications of malaria, ESR and cerebral malaria, Renal failure in malaria

1. Introduction

Known since millennia, malaria has played a major role in the history of mankind and has been a problem in India for centuries. It is often said that but for malaria, the history and geographical demarcations of our planet would have been different from what we have today. Malaria continues to wreak havoc on millions, particularly in the poorest parts of our world. Malaria has transmission in 107 countries containing 3 billion people and causing 1-3 million deaths each year. It is widespread in tropical and subtropical regions, including much of Sub-Saharan Africa, Asia and the Americas. Most deaths among the infected are caused by falciparum malaria. Hence it was declared to be the first priority tropical disease by the World Health Organisation (WHO)[1].

The considerable mortality and morbidity in falciparum malaria is due to its protean manifestations, multi organ involvement, delay in diagnosis and failure of administration of treatment promptly and adequately[2]. Added to this are the increasing problems of drug resistance of the parasite and insecticide resistance of

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the vectors. Although there are promising new control and research initiatives, malaria remains today, as it has been for centuries, a heavy burden on tropical communities, a threat to non endemic countries, and a danger to travelers. It has been especially of burden to the west coast of Karnataka, India especially during the monsoons. Hence an evaluation of the clinical profile of malaria for the emerging trends is appropriate in this context. The objective of this study was to document the various clinical manifestations, lab parameters and outcomes of falciparum malarial infection. Various complications encountered during the disease process were recorded and correlated with the parasitic index as a predictor of outcome and mortality.

2. Materials and methods

A total of 183 inpatients of Kasturba Hospital, Manipal aged above 18 years who were positive for *Plasmodium falciparum* were included in this study. This was conducted as a prospective study from May 2009 to January 2011 at Kasturba Hospital, Manipal, Karnataka, India. Our hospital, a tertiary referral centre is situated in an endemic area for malaria.

Ethical clearance was taken from the hospital committee and informed consent was taken from the patients or party as the situation demanded.

The methods used for diagnosis were immunofluoroscence (falcivax), quantitative buffy coat (QBC) and peripheral smear. Falcivax is a rapid, qualitative, two site sandwich immunoassay utilizing whole blood for the detection of *Plasmodium falciparum* specific histidine rich protein-2 (Pf. HRP-2) and *Plasmodium vivax* specific pLDH. The test can also be used for specific detection and differentiation of vivax and falciparum malaria in areas with high rates of mixed infections[3].

The QBC test is a new method for identifying the malarial parasite in the peripheral blood. It involves staining of the centrifuged and compressed red cell layer with acridine orange and its examination under UV light. It is fast, easy and claimed to be more sensitive than the traditional thick smear examination[4]. Out of the cases 4 people had vivax positivity in addition to falciparum.

Severe falciparum malaria was defined according to the one proposed by a working group convened by the WHO[5].

• Cerebral malaria-Unarousable coma not attributable to any other cause in a patient with falciparum malaria. The coma should persist for at least 30 min after a generalised convulsion to make the distinction from transient post ictal coma.

• Severe anemia-Normocytic anemia with hematocrit < 15% or hemoglobin <5 mg% in the presence of parasitemia >10 000/μL.

• Renal failure-defined as a urine out put of <400 mL in 24 h in adults or 12 mL/kg in 24 h in children, failing to improve after rehydration and serum creatinine of >3 mg%.

• Pulmonary edema or acute respiratory distress syndrome (ARDS).

• Hypoglycemia-defined as a whole blood glucose concentration of <2.2 mmol/L (40 mg%)

• Circulatory collapse or shock-hypotension (systolic blood pressure less than 50 mm Hg in children of 1-5 years or less than 70 mmHg in adults) with cold clammy skin or core-skin temperature difference of >10 °C.

• Spontaneous bleeding from gums, nose, and gastrointestinal tract etc. and or substantial laboratory evidence of disseminated intravascular coagulation.

• Repeated generalised convulsions more than 2 observed with in 24 h despite cooling of fever.

• Acidosis-defined as an arterial pH <7.25 or as a plasma bicarbonate concentration <15 mmol/L.

• Macroscopic hemoglobinuria-if definitely associated with acute malarial infection and not merely the result of oxidant anti malarial drugs in patients with erythrocyte enzyme defects *e.g.*, G6PD deficiency.

• Postmortem confirmation of diagnosis in fatal cases by histological examination of needle necropsy of the brain.

One or more of the above features in the presence of asexual parasitemia defines severe malaria.

Other manifestations of severe malaria which (according to the WHO document) do not in themselves define the condition in all geographical areas and age groups include the following:

• Impairment of consciousness less marked than unarousable coma.

• Prostration or weakness, so that the patient cannot sit or walk with no obvious neurological explanation.

• Very high parasite densities are associated with increased risk of severe disease. Most authorities would regard a parasitemia of more than 10% as indicating a potentially dangerous infection irrespective of the other features. (hyperparasitemia).

• Jaundice which is detected clinically or defined by a serum bilirubin concentration more than 3 mg %.

• Hyperpyrexia-rectal temperature more than 40 °C (sustained hyperpyrexia in severe malaria indicates a poor prognosis).

In severe malaria there is often evidence of multiple organ dysfunction and more than one of the above criteria are fulfilled

Statistical analysis was done using SPSS version 16.Tests used were independent paired *t* test, linear correlation and *Chi* square test. Results obtained were compared with a few similar studies.

3. Results

A total of 183 cases of Falciparum malaria were included in this study. The male sex group showed an increase in incidence (78%) compared to the female group. Of the 183 patients, 54% cases were between 21 and 50 years of age, with a peak of 22.5% patients in the second decade of life.

Most of the cases were between June and September corresponding with the monsoon season (Figure 1).This corresponded to two other studies carried out by Wasnik

PN *et al.* in 2012 and Koni MB in 2008 who had around 85% cases occurring during monsoons[6-8]. All cases were equally distributed in all classes of society. The nature of work and exposure to vector did not play a significant role. As our area contains a significant student community most of the cases were from the same group. 49.2% cases were from Udupi district, Karnataka in which our hospital is situated. The rest were cases with complications referred to our tertiary centre.

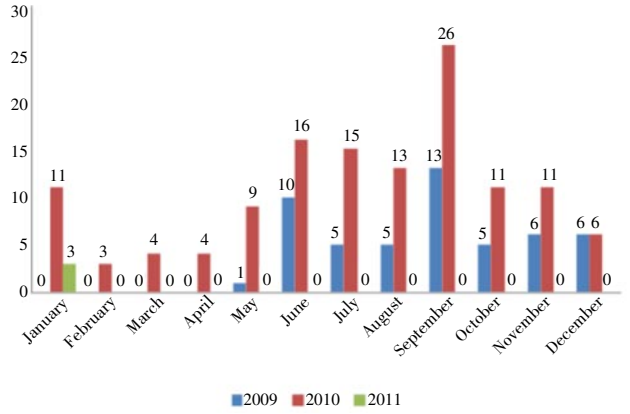

Figure 1. Monthly distribution of cases each year.

A total of 181 cases presented with fever as the initial symptom. Other major presenting symptoms in this study were jaundice, abdominal pain, vomiting, bleeding manifestations and head ache (Figure 2). A comparison of features with reference to other studies was as below (Table 1). And 48.1% cases were afebrile the day after admission. Average days of fever were one week. The presence of fever post admission was influenced by treatment which the patient had received outside. One case had hyperpyrexia (41.66 °C) which is a noted complication of falciparum malaria.

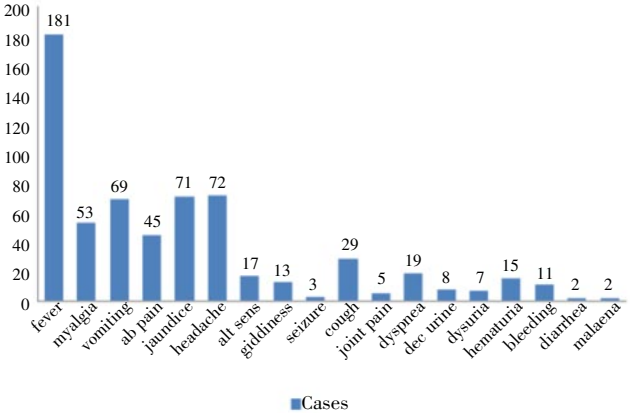

Figure 2. Symptoms encountered during the study.

Table 1

Comparison of clinical findings.

	Wasnik *et al*[6]	Koni MB *et al* Belgaum[7]	Gupta A *et al*[18]	Murthy *et al*[19]	Present study
Jaundice	35.0%	42.5%			38.79%
Splenomegaly		63.0%			42.62%
Hepatomegaly		65.0%			41.53%
Anemia	65.0%	57.5%	54.0%	86.48%	30.05%
Bleeding manifestations	57.5%	17.5%	21.8%	18.60%	27.23%

Twenty-seven cases had blood pressure below 100/60 mmHg and were given inotropic support. Out of this 14 cases fell into the WHO definition of shock which is a blood pressure below 70/60 mmHg[5].

Seventeen (9.28%) patients presented with altered sensorium. Out of these 3 persons went into unarousable coma. Altered sensorium and flapping tremors with deranged liver function tests were seen in 6 (3.27%) patients. Levels of ammonia were found to be above the upper range of normal (35 μmol/L) in these patients. Features of cerebral malaria in our study and in a similar study by Koni *et al*. were as follows. (Table 2)[7].

Table 2

Comparison of features of cerebral malaria.

	Koni *et al*. 2008 [7]	Present study
Incidence	57.5	9.28
Delirium	52.5	9.28
Coma	7.5	3.82
Headache	82.5	39.34
Meningeal signs	40.0	4.00

Cough was found in 37.5% of patients. Fifteen percent of the patients had early signs of respiratory insufficiency with tachypnoea, dyspnoea, shallow breathing, acidosis and bilateral chest signs like decreased air entry, crepitations and rhonchi. ARDS was the end result in 6 (3.27%) patients of which 3 (1.64%) were put on ventilator. All three expired eventually.

Three cases had hemoglobin less than 5 g/dL according to the WHO criteria for severe anemia in malaria (All three cases had hemoglobin levels less than 3 g/dL). And 28 cases (33.73%) had counts more than 15 000/μL. And 128 cases (69.9%) had platelet counts less than 1 lakh. Five cases had platelet counts less than 5 000/μL. And 30.05% had anemia according to WHO and 27.23% had deranged bleeding parameters. Twenty-eight cases required packed cell transfusion and 22 cases required platelet transfusion.

ESR levels were high in most patients (Figure 3). The relationship between WHO severity and ESR was significant with $P<0.003$. Severe cases had an average ESR of 60.10±42.30 as compared to the rest which had 38.83±31.87. The relationship of ESR and complications in malaria was found to be positive in several other studies[9]. The relationship between ESR and cerebral malaria was found to be not significant with a P value of 0.234.

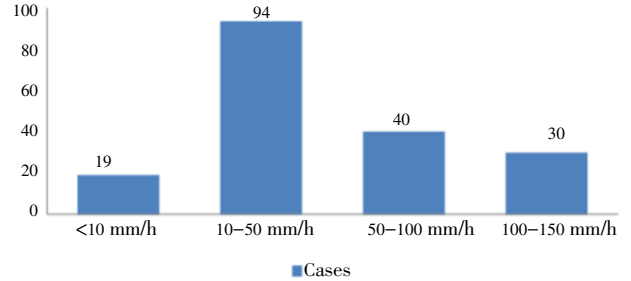

Figure 3. Ranges of ESR (mm/h) among the patients

Peripheral smear, QBC method and falcivax were the usual methods of diagnosis of falciparum malaria. Rate of

positivity hence sensitivity was more with falcivax where 13 cases were positive in which QBC was negative. The 5 cases of negative peripheral smear were positive by falcivax (Table 3). Sensitivity and specificity was not calculated using McNemar's formula as according to data, a gold standard investigation could not be established. Sixty-two cases (33.8%) had high parasitemia (4+) by QBC method (Figure 4). Distribution of parasitic index was as follows (Table 4).

Table 3

Results of diagnostic tests for falciparum malaria.

	Falcivax	MP QBC	Peripheral smear
Positive	161	165	178
Negative	10	18	5
Not available	12	0	0

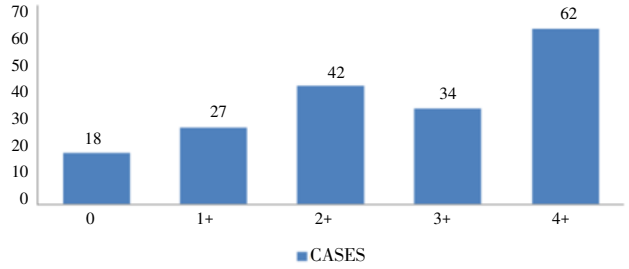

Figure 4. Ranges of parasitemia based on QBC method.

Table 4

Ranges of parasitic index among the cases.

Parasitic index	Cases
<0.5	69
0.5-1.0	13
1.0-5.0	37
5.0-10.0	4
>10.0	1

A creatinine value more than two is considered as renal failure according to the WHO criteria[5]. Thirty-five cases (19.13%) had renal failure. Eleven cases required dialysis. The relation between creatinine and death was significant with $P<0.0001$. Parasitic index and death according to Levenes test of equality of variance was significant with $P<0.01$ (Table 5). And 84 cases (45.9%) had elevated urea levels. In this study correlation between parasitic index and urea and creatinine values was significant according to Pearson's formula with a P value less than 0.000.

Table 5

Relationship between parasitic index, creatinine and death.

Death	Cases	Parasitic index	Creatinine
Yes	12	2.9±3.2	4.4±4.19
No	112	1.0±1.6	1.63±1.39

Contrary to popular belief hypoglycemia was not observed in this study during admission. Only 5 cases had random blood sugar values less than 60 mg/dL. None had less than 40 mg/dL according to the WHO severity criteria[5]. Also 15 patients were detected to be hyperglycemic out of who 9 were diabetics. The rest of the patients had been started on dextrose containing IV fluids prior to blood glucose being checked. This occurred probably due to over cautious management of anticipated hypoglycemia.

A total of 74.86% had normal sodium levels with 3.82% having hyponatremia. There was 1 case of hypernatremia. And 83.6% had normal potassium levels with 7.65% having hyperkalemia. All the cases of hyperkalemia were associated with renal failure. A total of 75 cases had a total bilirubin more than 3 g/dL which is taken as per the WHO severity criteria. Around 50% of the cases had deranged liver enzymes.

Out of the 15 cases of hematuria suspected clinically, 5 urine hemoglobin was sent all of which were positive. Out of the 5 cases of severe metabolic acidosis 3 were due to renal failure.

The relationship between age and death was not significant with $P=0.216$.

A few adverse effects of medications were recorded. There were two documented cases of cinchonism (tinnitus, giddiness) in whom quinine had been used. One case of lumefantrine induced psychosis and nightmares were observed. There was severe hemolysis in a patient induced by primaquine whose glucose six phosphate dehydrogenase (G6PD) level turned out to be normal. Levels of G6PD observed was as follows (Table 6)

Table 6

G6PD levels.

G6PD (U/g Hb)	Cases
Not available	95
Normal (6-12 U/g Hb)	85
Low (<6 U/g Hb)	3

Six blood cultures yielded positive results. There were two incidences of *Acinetobacter* and two of *E. coli* sepsis (grew in repeated cultures with evidence of sepsis in the patient). The other two grew Gram negative cocci which were probably contaminants (Table 7).

Table 7

Blood culture reports.

Culture reports	Cases
Sterile	147
Acinetobacter	2
E. coli	2
Gram negative cocci	2
Not available	30

Fourty-eight cases (26 %) required ICU care (Table 8). The mortality rate observed during the course of this study was 12.7%. The following were the causes of death found in the present study: 9 were due to multi organ dysfunction syndrome, severe metabolic acidosis. Three were due to isolated ARDS. One case of death was in a pregnant lady with high parasitemia due to ARDS and multi organ dysfunction syndrome.

Table 8

Requirement for ICU care.

ICU requirement		Cases
Dialysis		11
Inotropes		27
Ventilation	Venturi	15
	Non invasive ventilation	5
	Invasive ventilation	10
	ARDS	6
Altered sensorium		17
MODS		9

4. Discussion

This study was conducted on a prospective basis from May 2009 to January 2011. A total of 183 cases of *Plasmodium falciparum* were taken into account. The male group showed an increase in incidence (78%) as compared to the female group. And 54% of the cases were between 21 to 50 years of age, with a peak of 22.5% patients in the 2nd decade of life.

The male sex group showed an increase in incidence (78%) probably due to their occupation and hence proximity to vector contact. The relationship between age and death was not significant with P=0.216. One reason may be that children were not included and geriatric cases who are relatively immunosuppressed were less in number. Sex and age affect the incidence of falciparum malaria infection as they relate to the frequency of exposure and the development of immunity[10].

Falciparum malaria is equally distributed in all classes of society. As our population at Manipal is predominantly a student community a significant number of cases was from the same group. Quite a number of people who work indoors such as housewives were found to contract the disease. The nature of work and exposure to vector from outdoors did not play a significant role in the transmission of disease.

All the 181 cases presented with fever as the initial symptom. In a study conducted in the Royal Liverpool university hospital a majority of patients were afebrile through out the illness and presented with only vague constitutional symptoms and later as complications[11].

Seventeen cases (9.28%) patients presented with altered sensorium. Out of these 3 persons went into unarousable coma. The reported incidence of cerebral malaria in most studies is between 2%-55%. All the patients had fever, headache and altered sensorium of variable intensity during presentation. This hospital being a tertiary referral centre, the high incidence of cerebral malaria is expected. The comparison of the clinical profile of cerebral malaria in various studies showed a slightly lesser incidence[12]. The relationship between ESR and cerebral malaria was not significant (P=0.234) as found in other studies[13].

ARDS was the end result in 6 (3.28%) patients of which 3 (1.64%) were put on ventilator.

Twenty-eight cases (33.73 %) had counts more than 15,000. Gram negative sepsis which is thought to be a common event in complicated falciparum was rare in this study[14,15]. These infections could have occurred due to over zealous use of antibiotics and treatment in outside hospitals. Perhaps the falciparum malaria also made them more prone for the gram negative infections.

Anemia and abnormal bleeding parameters including deranged PT, APTT and thrombocytopenia were almost similar to other studies[16].

The rate of renal failure in our study was 32.7% which was almost similar to those conducted by Wasnik et al and Koni *et al.* which was 32.5% and 45% respectively[6,7]. The correlation between parasitic index and urea and creatinine values was significant according to Pearson's formula with P<0.000. In a similar study conducted in Indonesia, parasitic index was correlating with urea levels (P<0.05) and not correlating with creatinine levels (P>0.05)[17-19]. The relation between creatinine and death was significant with P<000.1 [17].

The following main conclusions were obtained from this study. Ninety-three (50.8%) cases had severe malaria according to the WHO criteria indicating most infections present with complications. The incidence of Gram negative sepsis was less. High parasite index and abnormal renal function tests are predictors of mortality and complications of disease.

Early diagnosis, anticipation of complications, close monitoring of vital parameters and combination therapy to over come drug resistance perhaps helps to contain the extent of mortality.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

Malaria plays a major role in the history of mankind and has been a problem in India for centuries. Malaria continues to wreak havoc on millions, particularly in the poorest parts of the world. Malaria has transmission in 107 countries containing 3 billion people and causing 1-3 million deaths each year. It is widespread in tropical and subtropical regions, including much of Sub-Saharan Africa, Asia and the Americas. Most deaths among the infected are caused by

falciparum malaria.

Research frontiers

A total of 183 inpatients of Kasturba Hospital, Manipal aged above 18 years who were positive for *Plasmodium falciparum* were included in this study. This was conducted as a prospective study from May 2009 to January 2011 at Kasturba Hospital, Manipal, Karnataka, India. The hospital is a tertiary referral centre is situated in an endemic area for malaria. The methods used for diagnosis were immunofluoroscence (falcivax), QBC and peripheral smear.

Related reports

In this study, 181 cases presented with fever as the initial symptom. However, in a study conducted in the Royal Liverpool University Hospital, majority of patients were afebrile throughout the illness and presented with only vague constitutional symptoms and later as complications. The comparison of the clinical profile of cerebral malaria in Laman *et al.* (2013) showed a slightly lesser incidence. The relationship between ESR and cerebral malaria was not significant as found in Ebuehi *et al.* (2012) study. The rate of renal failure in the present study was 32.7% which was almost similar to those conducted by Wasnik *et al.* (2012) and Koni *et al.* (2008) which were 32.5% and 45%, respectively.

Innovations and breakthroughs

This study reported that 50.8% of the cases had severe malaria according to the WHO criteria indicating most infections present with complications. The incidence of Gram negative sepsis was less. High parasite index and abnormal renal function tests are predictors of mortality and complications of disease. Early diagnosis, anticipation of complications, close monitoring of vital parameters and combination therapy to overcome drug resistance perhaps helps to contain the extent of mortality.

Applications

It may be significant to know the various clinical manifestations, lab parameters and outcomes of *Plasmodium falciparum* infection and correlate with the parasitic index as a predictor of outcome and mortality.

Peer review

This is a well-written manuscript on falciparum malaria in an endemic area of India. The results are interesting and suggested that 93 cases had severe malaria high parasite index and abnormal renal function tests are predictors of mortality and complications of disease.

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Hemophagocytic syndrome associated with severe *Plasmodium vivax* malaria in a child in Bikaner (northwestern India)

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Key words Childhood malaria; hemophagocytic syndrome; *Plasmodium vivax;* severe vivax malaria

Hemophagocytic syndrome (HPS) is a reactive disorder of the mononuclear phagocytic system, characterized by benign, generalized histiocytic proliferation, with marked hemophagocytosis in bone marrow[1]. Generally, HPS has been related with hematologic diseases, autoimmune diseases, or with various infections[1]. There are plenty of reports on hemophagocytic syndrome associated with *Plasmodium falciparum* monoinfetion[2–9], but reports on the association with *P. vivax* monoinfection are very scanty[9–14]. In most of these case reports, the diagnosis was made by peripheral blood smear (PBS) and rapid diagnostic test (RDT) without molecular diagnostic confirmation. Thus, there are always chances of species misidentification and missing the mixed infection thereby lacking authenticity. In this case report, the species diagnosis was confirmed by polymerase chain reaction (PCR) and possibilities of other diseases were ruled out by stringent laboratory and biochemical investigations to establish the firm association of hemophagocytic syndrome with *P. vivax* monoinfection.

Case report

On March 28, 2012, a female child was admitted with history of high grade fever with chills and rigor, emesis and abdominal pain since last seven days to the Department of Pediatrics, S.P. Medical College, Bikaner, Rajasthan, India. She had history of epistaxis and her parents also noticed petechiae spots on trunk and extremities since Day 1. There was no history of weight loss, bone pain, seizure, frequent infections, previous blood transfusion and any contact of tuberculosis. Past medical history and family history were also unremarkable. On physical examination, there was severe pallor, icterus, petechial spots, palpable cervical lymph nodes and hepatosplenomegaly (spleen 5 cm below the left costal margin). Vital signs were 100/60 mm Hg of blood pressure, 108/min of pulse rate, 36.9°C of temperature, and 26/min of respiration rate. Hematological findings revealed hemoglobin 5.2 g/dl, total leukocyte count 2100/mm^3, differential leukocyte count: 41% polymorphs, 53% lymphocytes, 3% monocytes, 3% eosinophils, and platelet count: 16,000/mm^3, hematocrit 22.6%, peripheral smear showing pancytopenia with all stages of *P. vivax* (density 8000/mm^3) (Fig. 1).

The RDT results, based on the detection of species-specific *Plasmodium* lactate dehydrogenase (LDH) (OptiMal test; Diamed AG, CressiersurMorat, Switzerland) and histidine rich protein-2 (HRP-2) (Falcivax test; Zephyr Biomedical Systems, Goa, India), were positive for *P. vivax* and negative for *P. falciparum*. The PCR study targeted against the 18S ribosomal RNA gene of the parasite and used 1 genus-specific 5′ primer and 2 species-specific 3′ primers in the same reaction mixture, confirmed the *P. vivax* monoinfection. The details are described in our previous studies[15].

Abnormal liver function tests were as follows: aspartate aminotransferase, 104 IU/L (reference: 5–45 IU/L); alanine aminotransferase, 106 IU/L (reference: 5–45 IU/L); total bilirubin, 3.3 mg/dl (reference: 0.2–1.13 mg/dl); and alkaline phosphotase, 856 IU/L (reference: 122–378 IU/L). Other investigations, results were as follows: serum ferritin level, 1070 ng/ml (reference: 15–332 ng/ml); fasting triglyceride levels, 297 mg/dl (reference: 30–160 mg/dl); serum fibrinogen levels, 102 mg/dl (reference: 233–496 mg/dl); and D-dimer assay, 25.7 µg/ml (reference: <1 µg/ml). Prothrombin time, activated partial thrombin time, renal function test, serum electrolytes and glucose-6-phosphate dehydrogenase enzyme levels were in normal range. Ultrasonography of the abdomen showed enlarged liver and spleen (12 cm). Immunohistochemistry including natural killer (NK) cells activity, soluble IL-2 and soluble CD25 measurement could not be done due to non-affordability by the patient.

Repeated culture (BACTEC- ALERT) of blood, urine and stool, and relevant serology tests for typhoid, Leptospira, rickettsia, hepatitis A/B/C viruses, HIV, infectious mononucleosis, dengue infection and fungal infec-

tion were all negative. Bone marrow was examined due to pancytopenia. Bone marrow aspirate smears showed normal cellularity and myeloid/erythroid ratio, adequate numbers of megakaryocytes/granulocytes/erythroid cells and sufficient hemosiderin particles. Intracellular parasites were rare but prominent hemophagocytic histiocytes were seen (Fig. 2). Diagnosis of malaria-associated HPS was made.

The child was treated with i.v. artesunate 2.4 mg/kg stat at 12 h, 24 h and then once daily for 2-days and then with oral artemether and lumefantrine for 3-days. Primaquine was given for 14 days as radical treatment. On Day 3, hematological findings were: hemoglobin 7.6 g/dl; total leukocyte count 7,600/mm^3; platelet count 146,000/mm^3 and peripheral smear showing disappearance of *P. vivax*. After 14 days of antimalarial medication, the child was discharged in hemodynamically stable state. Serum ferritin level and fibrinogen level were normalized during follow up.

Pancytopenia in a febrile patient may be the manifestation of bone marrow suppression induced by aplastic anemia, hematologic malignancies, metastatic cancer, infection, and or inflammation[16]. HPS, a rare cause of pancytopenia and fever, results from impaired functions of natural killer and cytotoxic T-cells and augmented activities of lymphocytes and histiocytes induced by overexpressed inflammatory cytokines such as tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and macrophage colony stimulating factor (M-CSF), soluble IL-2 receptor, IL-1 and IL-6, resulting in monocyte activation and leading to phagocytosis of hematopoietic cells[19–20]. Strikingly high levels of these cytokines have been observed in patients with malaria and could trigger HPS initiation[17–18]. Our patient presented with fever, hepatosplenomegaly, pancytopenia, hyperferritinemia, hypertriglyceridemia, hypofibrinogenemia and hemophagocytosis in the bone marrow, fulfilling the diagnostic criteria for HPS[17–18].

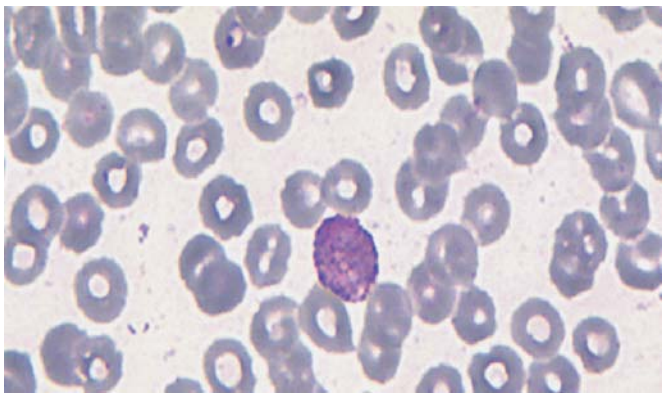

Fig. 1: Peripheral blood smear showing ring and schizont stages of *P. vivax.*

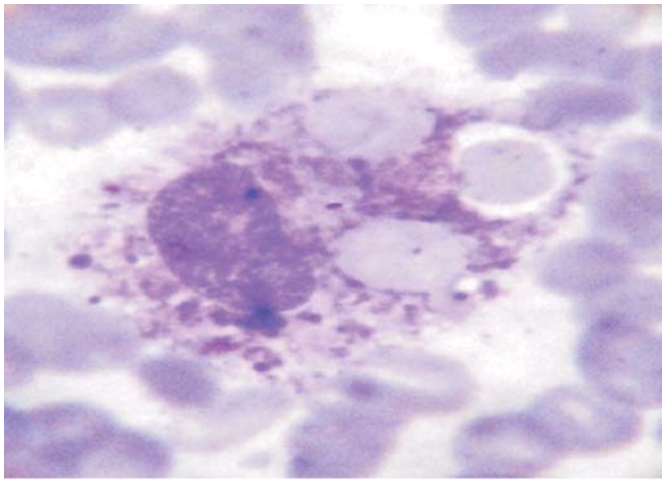

Fig. 2: Bone marrow aspiration smear showing hemophagocytosis (Leishman stain).

Severe *P. vivax* malaria has been recently reported in pediatric age from this community[19]. After extensive literature search, our case is the first PCR confirmed *P. vivax* associated HPS in childhood age. The etiological role of *P. vivax* monoinfection is suggested by the confirmation of species by PCR, ruling out the possibilities of other concurrent disease by scientific pattern and the total clinical and haematological recovery after antimalarial treatment. HPS is one of the causes of pancytopenia in these infections. Bone marrow examination is not usually undertaken for the purpose of diagnosis of malarial infection. Therefore, it is difficult to determine the prevalence of malaria complicated by HPS.

HPS could play a role in the pathogenesis of cytopenia observed during Plasmodia infestation. As its frequency has not been systematically studied during malaria, it is difficult to assume its pathophysiological consequences on prognosis. We recommend a marrow examination in malarial cases with severe or persistent decrease in hemoglobin or pancytopenia. However, it could be implicated in life-threatening complications due to the infection by Plasmodium species, justifying further studies on this syndrome to reduce disease burden.

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VERSITA

CASE REPORT

Vivax malaria presenting with cerebral malaria and convulsions

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Abstract

A patient was admitted with fever, vomiting, restlessness and convulsions. He was febrile and unconscious. Laboratory tests showed a low platelet count and ruled out enteric fever and dengue. His peripheral blood smear was positive for *Plasmodium vivax*. The presence of *P. vivax* monoinfection was confirmed by polymerase chain reaction and DNA sequencing. The report highlights the importance of considering the possibility of complications even in *P. vivax* malaria and formulation of strategies accordingly.

Keywords

Plasmodium vivax, cerebral malaria, India

A 20 year-old student having history of fever with chills and rigors for last four days and unconsciousness for last two hours was admitted to the intensive care unit of Tirathram Shah Charitable Hospital, Delhi, India in September 2008. He also had a history of convulsions (on two occasions), vomiting and restlessness during this period. There was no past history of convulsions.

On examination, he was febrile (102°F) and unconscious (Glasgow Coma Scale score: 8). His vital signs included pulse rate 92/min, respiratory rate 28/min and systolic blood pressure of 70 mm Hg. His spleen was palpable 2 cm below the costal margin. Neurological examination revealed normal reflexes and the fundus was normal. Laboratory investigations revealed the following: total serum bilirubin 2.56 mg/dl, serum ALT 162 IU/l, serum AST 75 IU/l, alkaline phosphatase 265 IU/l; total and differential leukocyte counts, renal function tests, blood glucose and serum electrolytes were within normal limits. CSF examination did not reveal any abnormality. Enteric fever and dengue were ruled out by blood culture, Widal Tube test and IgM ELISA, respectively. Platelet count was low (52000/µl). The electrocardiogram, electroencephalogram and computerized axial tomography were normal. The Giemsa stained blood smear was positive for *P. vivax* (parasite count: 48000 parasites/µl). Rapid diagnostic test (Falcivax®, Zephyr Biomedical Systems, India) was also positive for *P. vivax*.

The patient was treated with injectable artesunate, 60 mg at 0, 12, 24, 36, 48, 60 and 72 hours; followed by intramuscular chloroquine phosphate, 5 ml twice a day for three days. In addition, supportive therapy was given in the form of airway care, tepid sponging, hydration, antipyretics and third generation cephalosporin. He gained consciousness after three hours of admission. The blood pressure became normal on the second day of admission. Parasitaemia decreased to 2000 parasites/µl after 48 hours and was undetectable after 72 hours. Fever also subsided after three days. There were no neurological sequelae.

DNA extracted from blood using DNA extraction kit (Qiagen) was used for detection of malaria parasite as well as to rule out mixed infection by nested PCR assay as described by Rubio *et al*. (2002). The results confirmed *P. vivax* monoinfection (Fig. 1). For determination of sequence of small subunit ribosomal RNA (SSUrRNA) the part of SSUrRNA was amplified using *Plasmodium* specific primers rPLU1 and rPLU2 (Ng *et al*. 2008). PCR was done in 20 µl reaction volume containing 200 µmol/l of each dNTPs, 20 pmoles of each primer, 4.0 mM $MgCl_2$, 10 × Taq buffer and 1.0 U Taq polymerase (Bangalore Genei, Bengaluru, India) and 3 µl of genomic DNA as template following conditions as described earlier (Ng *et al*. 2008). Sequence of gel purified PCR product was determined using services of Macrogen (S. Korea); primers

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used for sequence determination are: rPLU1-5'-TCA AAG AAT AAG CCA TGC AAG TGA-3'; rPLU2-5'-TAC CCT GTT GTT GCC TTA AAC TCC-3'; rVIV1-5'CGC TTC TAG CTT AAT CCA CAT AAC TGA TAC-3'; rVIV2-5'-ACT TCC AAG CCG AAG CAA AGA AAG TCC TTA-3'. The sequence has been submitted to GenBank via accession no. GQ477744.

Vivax malaria has been considered to be benign for decades. However, various unusual presentations have been reported including respiratory, neurological complications and even death. Neurological syndrome has also been shown to be linked with *vivax* malaria (Kochar *et al*. 2007a). Seizures have been repeatedly observed in severe *vivax* malaria, and the cause has been attributed to the possible cerebral malaria, hypoglycemia, hyponatremia and lactic acidosis (Kochar *et al*. 2007b). Cerebral malaria in *P. vivax* infection has been linked to metabolic changes instead of sequestration (Rogerson and Carter 2008). However, there is another view which states the association of *vivax* malaria with blockade of the blood flow and production of TNF (Clark and Alleva 2009).

Thapa *et al*. (2007) reported two cases of *vivax* malaria from eastern India presenting with convulsions. Both the patients were unconscious at the time of admission. The patient had a low thrombocyte count. The association of platelet depletion and *vivax* malaria has been observed by various workers (Aggarwal *et al*. 2005, Rodríguez-Morales *et al*. 2006). A study from Mumbai has reported six cases of *vivax* malaria with thrombocytopenia, where the level of platelets ranged from 14000 to 92000/µl (Katira and Shah 2006). A recent study from India has reported cerebral malaria as a complication of *vivax* malaria in five out of 40 cases (Kochar *et al*. 2009).

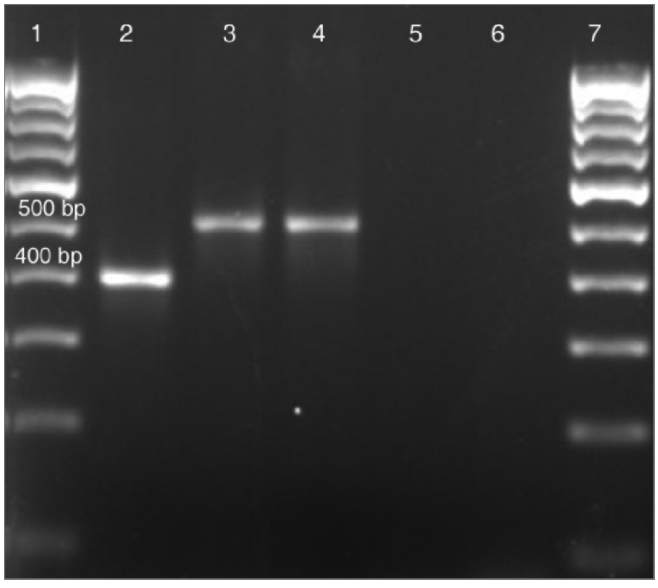

Fig. 1. PCR diagnosis of the patient sample. **1.** Marker = 100 bp ladder. **2.** Positive *P. falciparum* control. **3.** Positive *P. vivax* control. **4.** Patient sample (amplified using *P. vivax* primers). **5.** Patient sample (amplified using *P. falciparum* primers). **6.** Negative control. **7.** Marker = 100 bp ladder

Present report highlights the importance of suspecting complications even in *P. vivax* malaria. This is particularly important since there is a tendency to suspect *P. falciparum* infection whenever such situation arises, on the basis of rapid test using HRP II antigen.

P. vivax strains from India are in general sensitive to chloroquine but resistance has been recorded in some reports (Dua *et al*. 1996, Singh 2000). A therapeutic efficacy study from western India has also shown chloroquine resistance in *vivax* malaria (Srivastava *et al*. 2008). Whether chloroquine resistance is a contributing factor for complications needs to be studied.

The report highlights that *P. vivax* infection can present with altered mental state and convulsions apart from the usual symptoms. Areas with higher *P. falciparum* proportion are classified as high risk and priority is given for introduction of new, rapid and better diagnostic tools and effective drugs. The observation of these clinical presentations are warning signals for better vigilance in *P. vivax* areas which are at present classified as low risk.

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BRIEF RESEARCH REPORT Open Access

Cross-sectional survey of malaria prevalence in tsunami-affected districts of Aceh Province, Indonesia

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Abstract

Background: Malaria is endemic to Indonesia. However, there are few prevalence data available from Aceh Province because of the long-standing separatist conflict and decentralization of the public health system. The Mentor Initiative, which specializes in malaria control in humanitarian emergencies, was one of the non-governmental organizations to respond to the 2004 Indian Ocean tsunami in Aceh. Data on malaria prevalence were gathered to guide and evaluate programmatic efforts.

Findings: The Mentor Initiative conducted community-based malaria prevalence surveys in 2005 and 2006 in five districts along the tsunami-affected western coastline. A total of 11,763 individuals in 3,771 households were tested. The overall slide positivity rate in 2005 and 2006 for all *Plasmodium* species was 2.1% (n = 252, 95% CI 1.9%-2.4%). Slide positivity rates ranged from 0 to 55% among villages. Overall, 57% of the 252 cases were infected with *P. falciparum* (n = 144, 95% CI 51.0%-63.3%), and 40.1% were infected with *P. vivax* (n = 101, 95% CI 34.0%-46.1%), with 0.03% (n = 7, 95% CI 0.8%-4.8%) being mixed infections. Males were significantly more likely to be affected than females (2.8% vs 1.5%, $p < 0.01$). Infection was more common in those over the age of 5 (2.3% vs. 0.6%, $p < 0.01$).

Conclusions: Local prevalence data are needed to design effective community-based malaria control programs, as endemicity varies greatly within districts. Certain villages were found to be hyperendemic, with slide positivity rates far higher than average in Indonesia. There is a need for ongoing malaria surveillance in Aceh Province to monitor prevention and treatment efforts.

Introduction

Malaria is endemic throughout much of Indonesia. The Indonesian government reported a countrywide malaria prevalence of 850 per 100,000 in 2001 [1]. The World Health Organization's (WHO) 2008 World Malaria Report stated that 37% of Indonesia's population lived in a high transmission area (≥ 1 case/1,000), 14% lived in a low transmission area (0-1 cases/1,000), and 50% lived in a malaria-free area [2]. Endemicity tends to be higher on the more heavily forested outer islands. Approximately 46% of malaria infections in Indonesia are due to *Plasmodium falciparum* [3].

Sumatra, one of Indonesia's outer islands, was severely affected by the 2004 Indian Ocean tsunami, with much of the destruction located in the northern Aceh Province. Aceh Province had been quite isolated prior to the tsunami because of a long-standing separatist conflict, and therefore relatively little malaria prevalence information is publicly available. The South-East Asia Regional Office of the WHO reported that Aceh had low to moderate endemicity in 2005 [4]. The Indonesian Ministry of Health reported fewer than 1,000 blood smears and rapid diagnostic tests (RDTs) in Aceh were positive for malaria in 2002, but was unable to provide a slide positivity rate [5]. Between 5,000 and 9,999 "clinically confirmed" cases were reported in Aceh during the same year, but "clinical confirmation" is known to be inaccurate [5]. The World Malaria Report found that much of Sumatra had a prevalence ranging from 1 to

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100 reported cases per 1,000, but reported no data from Aceh Province [1].

The Mentor Initiative, a non-governmental organization specializing in malaria prevention and treatment in complex emergencies, was one of many humanitarian groups that provided assistance in Indonesia after the tsunami. Given the paucity of baseline data on malaria prevalence, and the need to set prevention priorities and monitor program effectiveness, the Mentor Initiative conducted two community-based malaria prevalence surveys in tsunami-affected subdistricts of Aceh Province in 2005 and 2006. The data presented here are the first published in a peer-reviewed journal from Aceh, and this is among the few publications of the state of malaria prevalence in the post-tsunami setting.

Methods

Survey protocol

Although rain can occur episodically throughout the year, the rainy season in Aceh usually lasts from September to February. Malaria transmission is thought to vary seasonally in Aceh, peaking during the rainy season. Surveys were conducted during the dry season between May and July 2005 and again between April and July 2006.

Aceh province is divided into 21 districts. Five tsunami-affected districts were identified for the community-based malaria prevalence surveys conducted as part of a broader malariometric survey: Aceh Barat, Aceh Jaya, Nagan Raya, Woyla Barat, and Simeulue. Selection was based, in part, on predicted malaria burden, and also on the area's accessibility and security. In 2005, seven subdistricts of the aforementioned districts were surveyed: Seulimeum, Lamno, Woyla Induk, Woyla Timur, Beutong, Simeulue Timur, and Teupah Selatan. In 2006, a follow-up survey was conducted in these same subdistricts (except that Sungai Mas in Woyla Barat district was included and Simeulue Timur was excluded). Both years' data are presented in this article.

Within each subdistrict, Probability Proportional to Size cluster sampling was used to select villages. Within each village, households were selected by simple random sampling. The target sample size was 10% of the population of each subdistrict. Written consent was obtained from the head of each household, and all members of the household were surveyed.

In each subdistrict, survey data were collected by two teams of three health care providers (usually nurses) from the local clinics (*puskesmus*), and data collection was overseen by a supervising physician from The Mentor Initiative. The survey team was trained to collect survey data, perform the RDT, and prepare the thick and thin blood smears for microscopy. All previously untreated patients who tested positive for malaria were given appropriate therapy according to local and Roll Back Malaria in Complex Emergency guidelines.

Laboratory diagnosis

Microscopy was considered the gold standard for diagnosis of malaria infection. Slides were prepared by survey staff and read by a Mentor-trained laboratory technician as well as two technicians from the Provincial Health Office. Systematic external quality controls were conducted to ensure accuracy of the blood smear readings by the survey team. The prevalence survey was conducted contemporaneously with a second study designed to field test RDTs. In 2006, the RDT used was Falcivax, which detects *P. falciparum, P. vivax*, and mixed infections. During 2006, the blood smears obtained in the subdistricts Woyla Induk and Woyla Timur were contaminated during preparation and rendered unreadable. Therefore, in these two subdistricts, RDT results are reported in lieu of blood smear results.

Statistical analysis

Data were entered into Epi Data and analyzed using Epi Info. Pearson's chi-square test was used to test for significance, where appropriate. P values of < 0.05 were considered statistically significant.

Ethical considerations

Approval to carry out the survey was obtained by The Mentor Initiative from the Provincial Health Office and each District Health Office in Aceh Province. The Institutional Review Board of Mount Sinai School of Medicine approved the retrospective data analysis.

Results

A total of 3,771 households and 11,763 individuals were surveyed in 220 villages in 2005 and 2006. Details of individuals, households, and villages surveyed are provided in Table 1. Overall, 43.6% (n = 5,130) of those surveyed were male. Males were significantly more likely

Table 1 Number of villages, households, and individuals surveyed in each subdistrict

	Villages		Households		Individuals	
Subdistrict	**2005**	**2006**	**2005**	**2006**	**2005**	**2006**
Seulimeum	19	24	285	480	903	1791
Lamno	24	24	360	408	1070	944
Woyla Induk	20	22	397	308	1184	1012
Woyla Timor	13	13	240	104	775	257
Sungai Mas	-	9	-	81	-	219
Beutong	10	14	250	350	1189	1026
Simeulue Timur	10	-	150	-	458	-
Teupah Selatan	10	8	150	208	392	543
Total	**106**	**114**	**1832**	**1939**	**5971**	**5792**

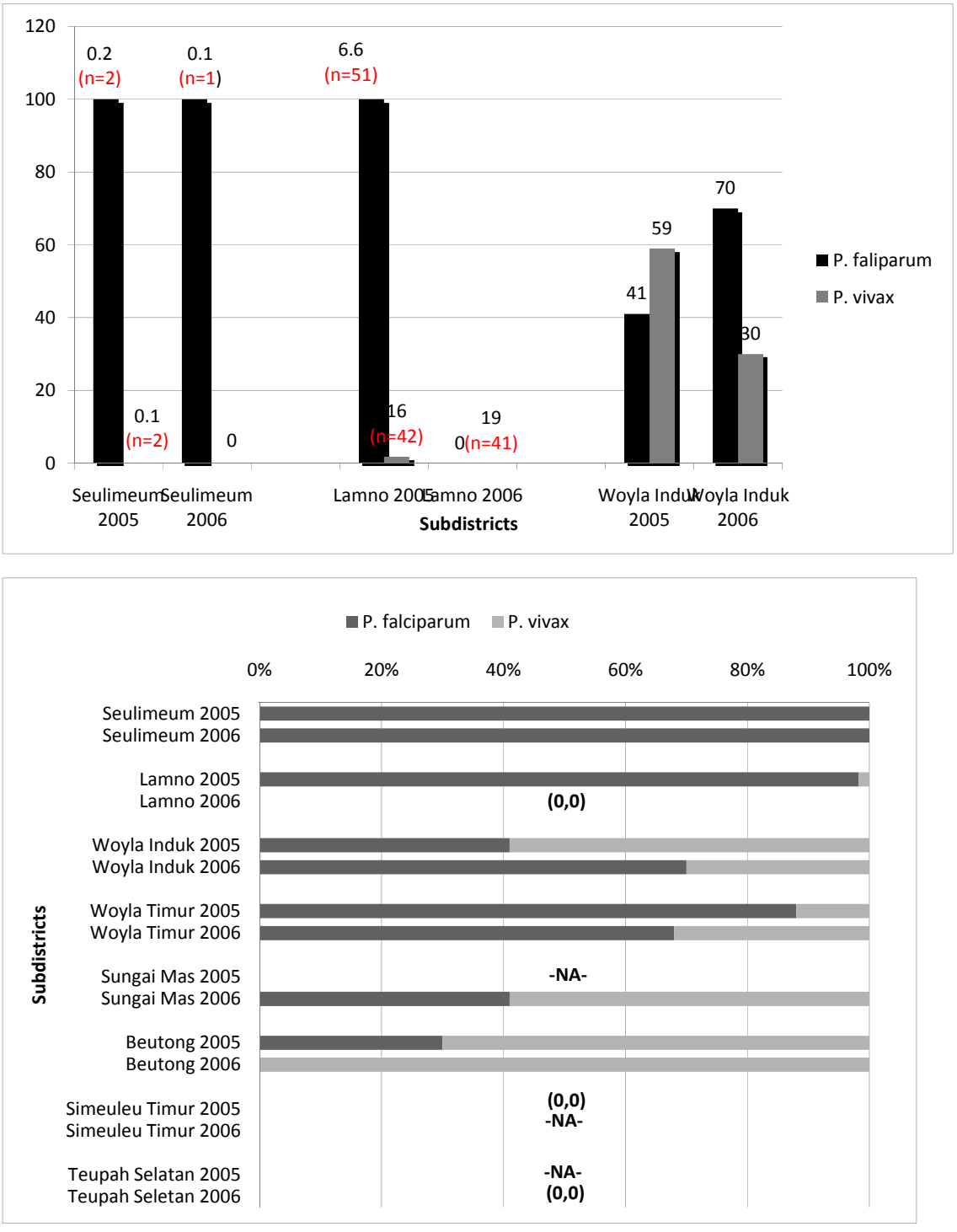

Figure 1 Positive blood smears. **(a)** Percent of positive blood smears in 2005 and 2006, by subdistrict. **(b)** Percent of blood smears positive for *P. falciparum* and *P. vivax* in each subdistrict.

to have parasitemia than females (2.8% vs. 1.5%, $p < 0.01$). Of the population surveyed, 12.3% ($n = 1,447$) was less than or equal to 5 years old and 23.9% ($n = 2,817$) less than or equal to 10. The mean age could not be calculated as data were recorded categorically (by age group). Parasitemia was less common in children less than or equal to 5 years old compared with older children and adults (0.6% vs. 2.3%, $p < 0.01$). Malaria

infection was significantly more common among actively febrile patients (16% vs. 1.7%, $p < 0.01$).

Prevalence data and slide positivity rates for *Plasmodium falciparum* versus *P. vivax* are presented in Figure 1a, b. The overall slide positivity rate in both 2005 and 2006 for all *Plasmodium* species was 2.1% (n = 252, 95% CI 1.9%-2.4%), but it was much higher in the subdistricts of Woyla Timur and Sungai Mas (Figure 1a). Two species of malaria parasites, *Plasmodium falciparum* and *P. vivax*, were identified. Overall, *P falciparum* accounted for 57.1% of infections (n = 144, 95% CI 51.0%-63.3%) and *P. vivax* for 40.1% (n = 101, 95% CI 34.0%-46.1%), with 0.03% (n = 7, 95% CI 0.8%-4.8%) being mixed infections. The proportion of *P. falciparum* and *P. vivax* varied among subdistricts. As displayed in Figure 1b, *P. falciparum* was more common in the subdistricts of Woyla Induk and Woyla Timur, whereas *P. vivax* predominated in Beutong and Sungai Mas. As discussed in the methods section, the RDT Falcivax, which can distinguish among *P. falciparum, P. vivax*, and mixed infections, was substituted for blood smear results in 2006 in the subdistricts of Woyla Induk and Woyla Timur because of contamination of the slides.

Within each subdistrict, the intervillage positivity rates varied widely (see Table 2). In the subdistricts of Woyla Induk and Woyla Timur, some villages in 2005 had rates as high as 13% and 31%, respectively. In 2006, villages in the subdistricts of Woyla Induk, Woyla Timur, and Sungai Mas had positivity rates of 26%, 41%, and 55%, respectively.

Discussion

This is the first peer-reviewed study of malaria prevalence in post-tsunami Aceh Province, Indonesia. Most of what is known about malaria prevalence in Indonesia is based in regions of the country outside of Aceh and therefore has a limited application in Aceh itself [2]. Our results show great variability of malaria prevalence among subdistricts, and even more so among villages, with small areas of transmission rates approaching those found in sub-Saharan Africa [1].

Because of the limited data available prior to the 2004 Indian Ocean tsunami, it is difficult to compare malaria prevalence before and after the disaster. The post-tsunami conditions were thought to be high risk for a malaria epidemic, with massive displacement of a semi- or non-immune population, temporary and crowded housing, standing water with the creation of new vector breeding areas, and disruption of the public health and health care system. These data strongly suggest that an epidemic did not occur and that malaria transmission was kept at low rates in most areas during the 2 years following the disaster. However, without pre-tsunami data, the impact of the massive malaria control efforts by various national and international agencies cannot be determined from this study.

Our survey found that Aceh males are more likely to have parasitemia than females, which is consistent with the Indonesian Ministry of Health's report that death rates for men are 11 per 100,000 compared with 8 per 100,000 for females [3]. This may reflect variable exposure to mosquitoes through differing occupational or recreational activities. We also found great variation in malaria prevalence rates between and within subdistricts. Several studies have examined the spatiotemporal variations in malaria epidemic risk, particularly in Africa [6,7]. The wide variability in endemicity among villages likely reflects the microclimate of that village, with variable forest cover and water sources. Factors that serve as important determinants of the severity of malaria transmission include altitude, forest cover, soil water holding capacity, and precipitation. Despite their close proximity, differences in these risk factors among the subdistricts might impact the intensity of malaria transmission. Incorporating such information into future malaria prevention and control programs could serve to predict the likelihood of severe malaria epidemics and allow for local or community-based control efforts to be tailored appropriately.

Table 2 Prevalence range among villages within each subdistrict

	Prevalence range among villages (%)	
Subdistrict	2005	2006
Seulimeum	0-2.2	0-1.3
Lamno	1-1.5	0
Woyla Induk	0-13	0-26
Woyla Timur	0-31	0-41
Sungai Mas	n/a	0-55
Beutong	0-7	0-4
Simeulue Timur	0	n/a
Teupah Selatan	0	0

List of abbreviations

CI: confidence interval; WHO: World Health Organization; RDT: rapid diagnostic tests.

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Authors' contributions

DM helped conceive, design, and coordinate the study in the field. SH performed the statistical analysis and co-wrote the manuscript. BH performed the statistical analysis and co-wrote the manuscript. RA conceived of the study, participated in its design and coordination and reviewed the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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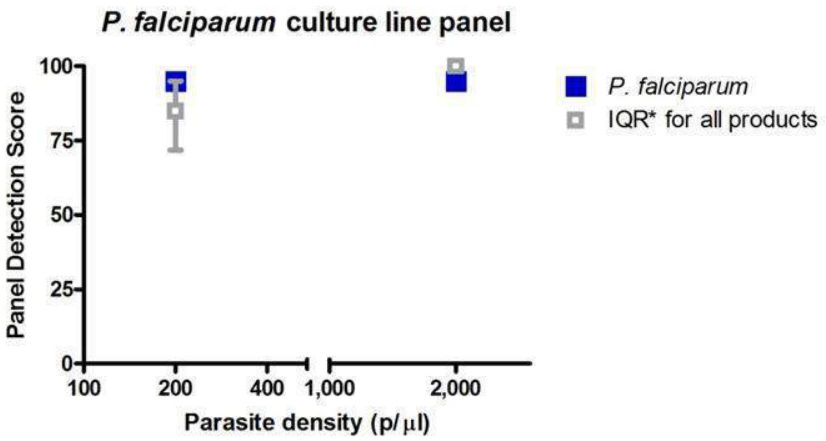
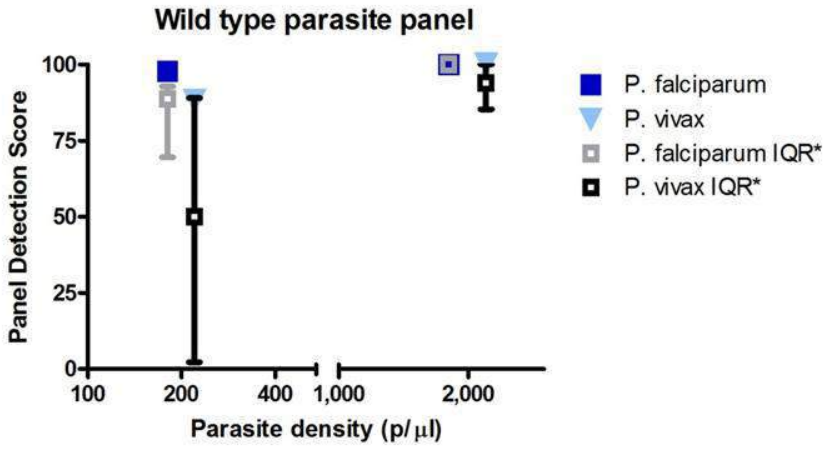


* IQR: interquartile range with median (square) for all products tested



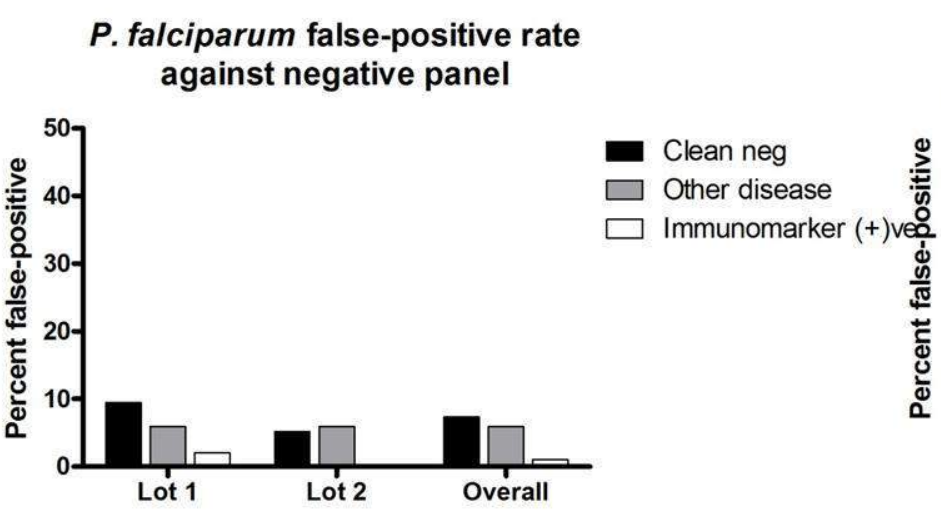
* IQR: interquartile range with median (square) for all products tested

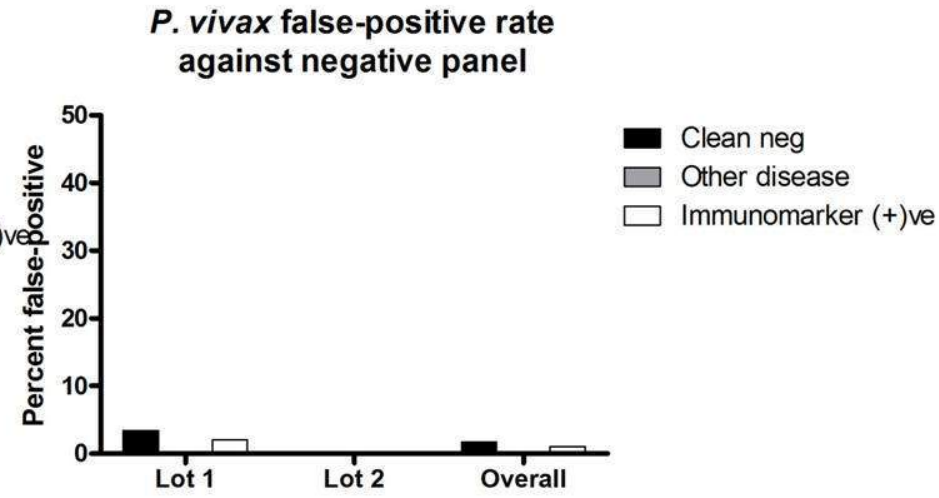
Panel Detection Score (PDS) and Sample Size (n)

		200 p/µl	2000 p/µl
All Products (PDS)	Q1 (25th %ile)	71.3	100.0
	Median	85.0	100.0
	Q3 (75th %ile)	95.0	100.0
	Your product scored:	95.0	95.0
	n	20.0	20.0

Panel Detection Score (PDS) and Sample Size (n)

			200 p/µl	2000 p/µl
All Products (PDS)	*P. falciparum*	Q1 (25th %ile)	69.6	99.0
		Median	88.8	100.0
		Q3 (75th %ile)	92.9	100.0
		Your product scored:	98.0	100.0
		n	98.0	98.0
All Products (PDS)	*P. vivax*	Q1 (25th %ile)	2.2	85.3
		Median	50.0	94.1
		Q3 (75th %ile)	89.0	100.0
		Your product scored:	88.2	100.0
		n	34.0	34.0





Percent false-positive

	Clean neg	Other disease	Immunomarker (+)ve
Lot 1	9.5	5.9	2.0
Lot 2	5.2	5.9	0.0
Overall	7.3	5.9	1.0

n

	Clean neg	Other disease	Immunomarker (+)ve
Lot 1	116.0	34.0	2.0
Lot 2	116.0	34.0	0.0
Overall	232.0	68.0	1.0

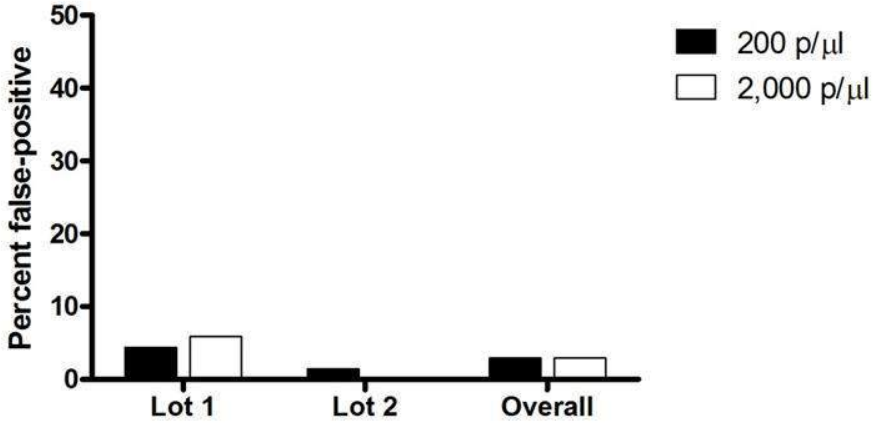
Percent false-positive

	Clean neg	Other disease	Immunomarker (+)ve
Lot 1	3.5	0.0	2.0
Lot 2	0.0	0.0	0.0
Overall	1.7	0.0	1.0

n

	Clean neg	Other disease	Immunomarker (+)ve
Lot 1	116.0	34.0	50.0
Lot 2	116.0	34.0	50.0
Overall	232.0	68.0	100.0

P. falciparum false-positive rate against *P. vivax* panel



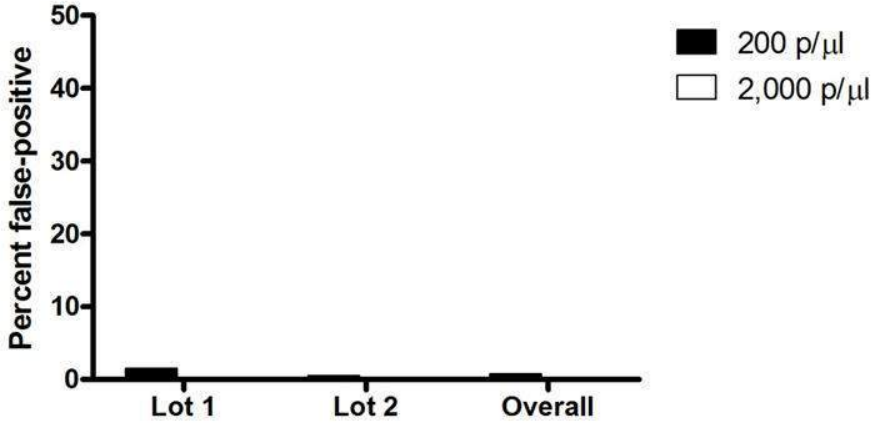
Percent false-positive

	200 p/µl	2,000 p/µl
Lot 1	4.4	5.9
Lot 2	1.5	0.0
Overall	2.9	2.9

n

	200 p/µl	2,000 p/µl
Lot 1	68.0	34.0
Lot 2	68.0	34.0
Overall	136.0	68.0

P.vivax false-positive rate against *P. falciparum* panel



Percent false-positive

	200 p/µl	2,000 p/µl
Lot 1	1.5	0.0
Lot 2	0.5	0.0
Overall	0.8	0.0

n

	200 p/µl	2,000 p/µl
Lot 1	196.0	98.0
Lot 2	196.0	98.0
Overall	392.0	196.0

Performance of individual tests in Lot 1 against the wild type panel

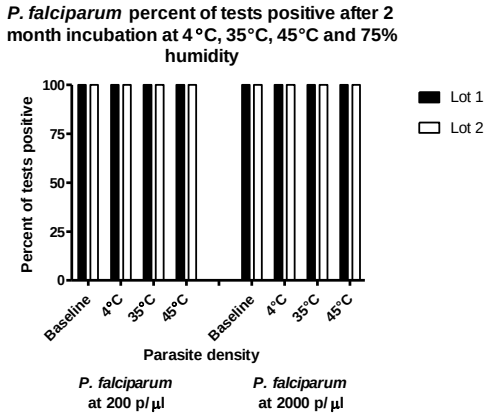
Type of wild type panel

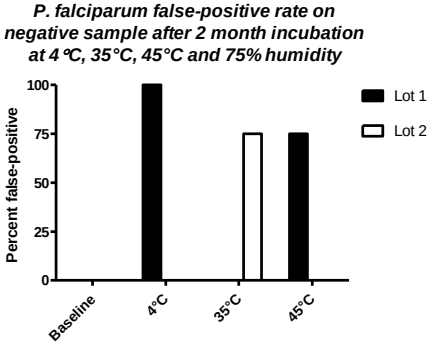
Test result	P. falciparum (200p/ μl)	P. vivax (200 p/ μl)	Plasmodium Negative
Pf (+ve)	194.0	3.0	14.0
Pf (-ve)/Pv (+ve)	0.0	65.0	3.0
Negative	2.0	0.0	183.0
Invalid Test	0.0	0.0	0.0
Total	196.0	68.0	200.0

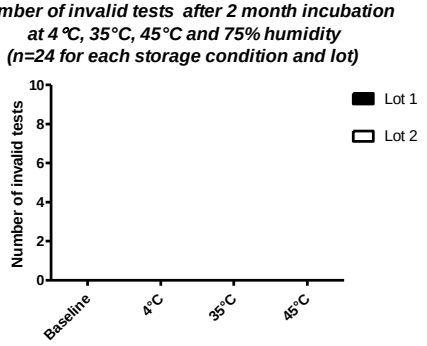
Performance of individual tests in Lot 2 against the wild type panel

Type of wild type panel

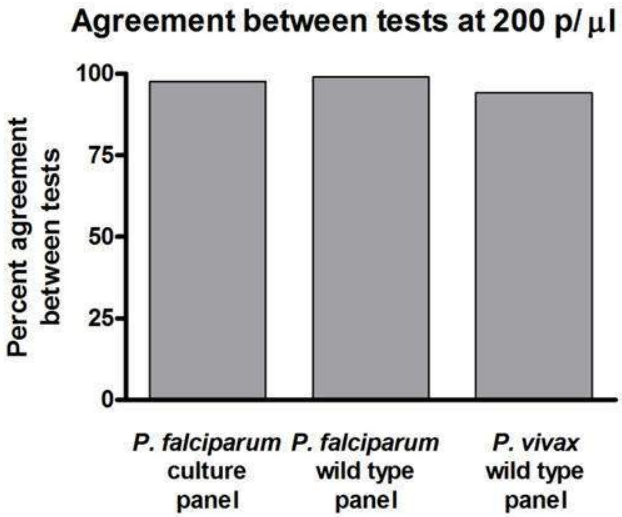
Test result	P. falciparum (200p/ μl)	P. vivax (200 p/ μl)	Plasmodium Negative
Pf (+ve)	194.0	1.0	8.0
Pf (-ve)/Pv (+ve)	0.0	64.0	0.0
Negative	2.0	3.0	192.0
Invalid Test	0.0	0.0	0.0
Total	196.0	68.0	200.0

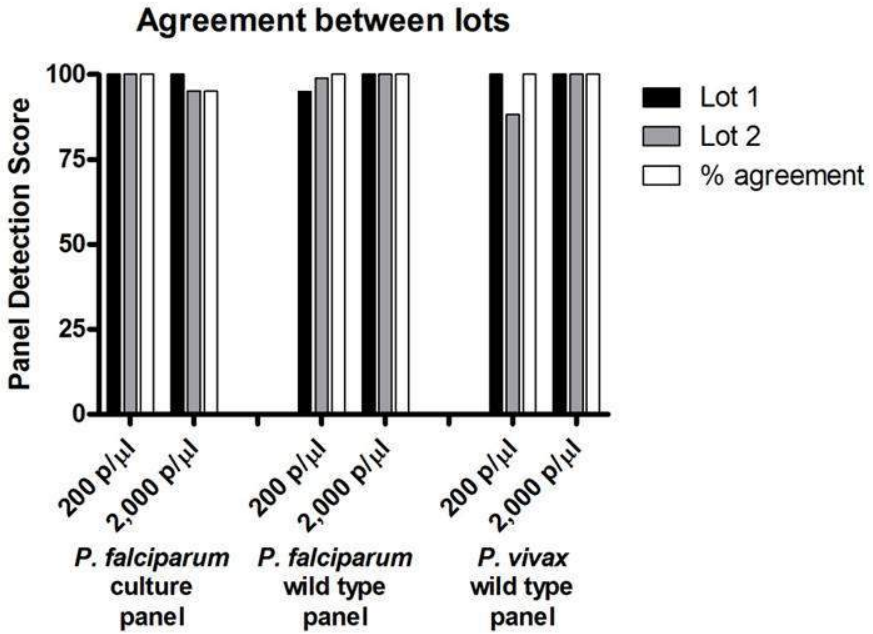






		Percent of tests positive		Percent false-positive	
		P. falciparum (200p/ µl)	P. falciparum (2000p/ µl)	Negative Samples	Number of Invalid Tests (n=24)
Lot 1	Baseline	100.0	100.0	0.0	0.0
	4°C	100.0	100.0	100.0	0.0
	35°C	100.0	100.0	0.0	0.0
	45°C	100.0	100.0	75.0	0.0
Lot 2	Baseline	100.0	100.0	0.0	0.0
	4°C	100.0	100.0	0.0	0.0
	35°C	100.0	100.0	75.0	0.0
	45°C	100.0	100.0	0.0	0.0
n					
Lot 1	Baseline	15.0	5.0	4.0	
	4°C	15.0	5.0	4.0	
	35°C	15.0	5.0	4.0	
	45°C	15.0	5.0	4.0	
Lot 2	Baseline	15.0	5.0	4.0	
	4°C	15.0	5.0	4.0	
	35°C	15.0	5.0	4.0	
	45°C	15.0	5.0	4.0	



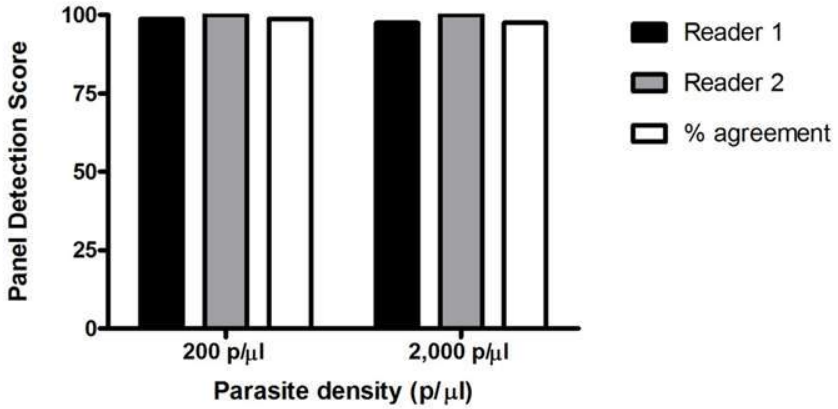


	200 p/μl	n
P. falciparum culture panel	97.5	40.0
P. falciparum wild type panel	99.0	196.0
P. vivax wild type panel	94.1	68.0

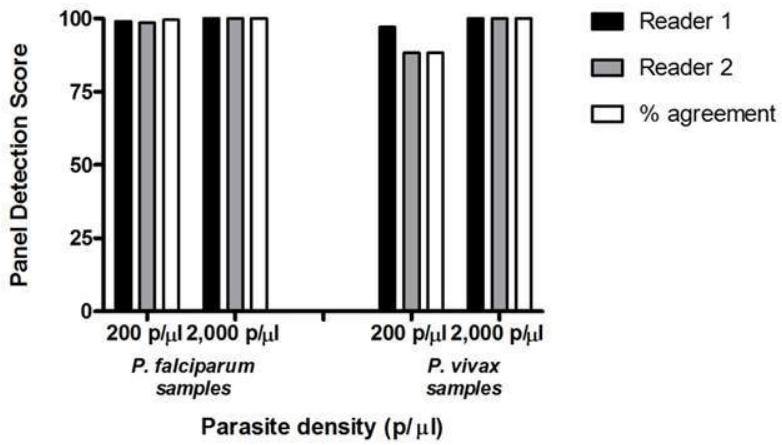
Panel Detection Score

		P. falciparum culture panel		P. falciparum wild type panel		P. vivax wild type panel	
		200 p/ul	2000 p/ul	200 p/ul	2000 p/ul	200 p/ul	2000 p/ul
	Lot 1	100.0	100.0	98.0	100.0	100.0	100.0
	Lot 2	95.0	95.0	99.0	100.0	88.2	100.0
	% agreement	100.0	95.0	100.0	100.0	100.0	100.0
n	Lot 1	20.0	20.0	98.0	98.0	34.0	34.0
n	Lot 2	20.0	20.0	98.0	98.0	34.0	34.0
n	No. in agreement	19.0	20.0	96.0	98.0	30.0	34.0

Agreement between readers: *P. falciparum* culture panel



Agreement between readers: wild type parasite panel



Positivity

	Reader 1	Reader 2	% agreement
200 p/µl	98.8	100.0	98.8
2,000 p/µl	97.5	100.0	97.5

n

	Reader 1	Reader 2	No. in agreement
200 p/µl	80.0	80.0	80.0
2,000 p/µl	40.0	40.0	40.0

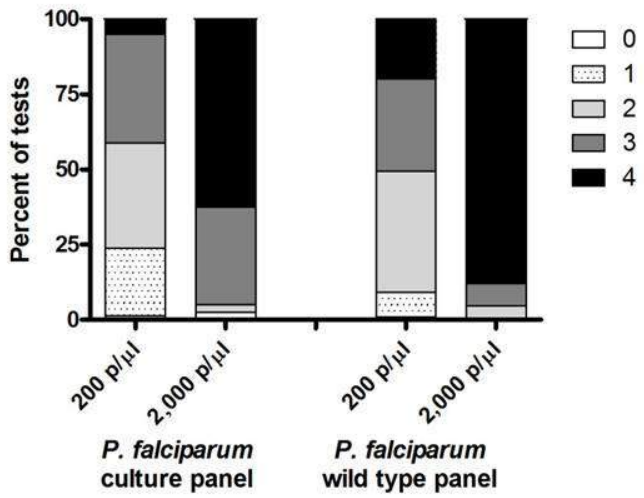
Positivity

		Reader 1	Reader 2	% agreement
P. falciparum samples	200 p/µl	99.0	98.5	99.5
	2,000 p/µl	100.0	100.0	100.0
P. vivax samples	200 p/µl	97.1	88.2	88.2
	2,000 p/µl	100.0	100.0	100.0

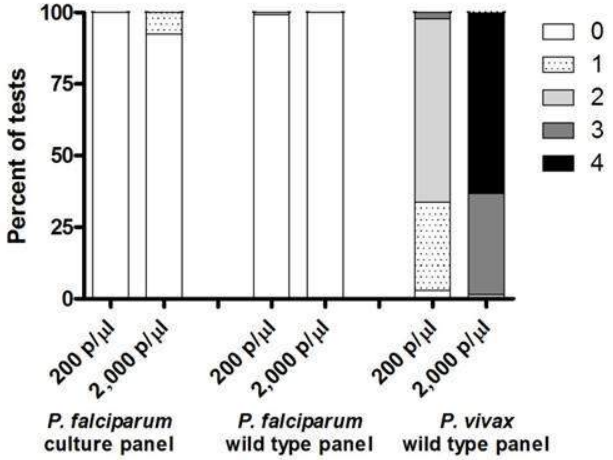
n

		Reader 1	Reader 2	No. in agreement
P. falciparum samples	200 p/µl	392.0	392.0	392.0
	2,000 p/µl	196.0	196.0	196.0
P. vivax samples	200 p/µl	136.0	136.0	136.0
	2,000 p/µl	68.0	68.0	68.0

Distribution of *P. falciparum* test band intensity



Distribution of *P. vivax* test band intensity



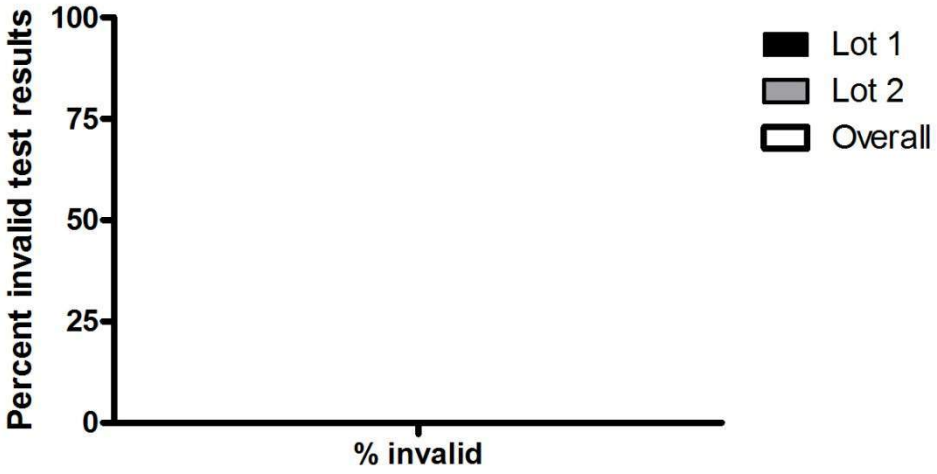
Band intensity (percent of tests)

		0	1	2	3	4	n
P. falciparum **culture panel**	**200 p/µl**	1.3	22.5	35.0	36.3	5.0	80.0
	2,000 p/µl	2.5	0.0	2.5	32.5	62.5	40.0
P. falciparum **wild type panel**	**200 p/µl**	1.0	8.2	40.3	30.6	19.9	3.9
	2,000 p/µl	0.0	0.0	4.6	7.7	87.8	196.0

Band intensity (percent of tests)

		0	1	2	3	4	n
P. falciparum **culture panel**	**200 p/µl**	100.0	0.0	0.0	0.0	0.0	80.0
	2,000 p/µl	92.5	7.5	0.0	0.0	0.0	40.0
P. falciparum **wild type panel**	**200 p/µl**	99.2	0.0	0.8	0.0	0.0	392.0
	2,000 p/µl	100.0	0.0	0.0	0.0	0.0	196.0
P. vivax **wild type panel**	**200 p/µl**	2.9	30.9	64.0	2.2	0.0	136.0
	2,000 p/µl	0.0	0.0	1.5	35.3	63.2	68.0

Invalid test results during testing against culture and wild type panels



Percent invalid test results

	Lot 1	Lot 2	Overall
% invalid	0.0	0.0	0.0

n

	Lot 1	Lot 2	Overall
Number of Tests	456.0	456.0	912.0

Ease of Use

Blood safety[1]: 2
Instruction quality[2]: 2
Timed steps required: 1
Total time to result: 20 mins
Blood transfer device: pipette
Format: cassette
Language(s) of instructions: English
Items included in package: Cassette, pipette, alcohol swab, buffer, desiccant (no color change)

Comments: n/a

[1] Blood safety scoring scheme: Mixing wells involved (Y=0/N=1); Retractable needle (No retract. needle = 0, retract. needle =1); Strip exposed: not within card/cassette (Exposed = 0/ Covered = 1)
[2] Instruction quality scoring scheme: No Pictures / diagrams 0; Pictures /diagrams of result 1; Pictures / diagrams of results and method 2; Qualitative assessment (0 poor - 2 good)

Evaluation of FalciVax Kit Manufactured by M/S Zephyr Bio-Medical, Verna, Goa.

Testing Laboratory: Malaria Research Centre (ICMR), DHS Building, Campal, Panaji, Pin-403 001, Goa, India.

Name of the Product: FalciVax

Type of Product: Immunochromatic Rapid Diagnostic kit for malaria parasites *Plasmodium vivax* and *Plasmodium falciparum*

Period of study: 29th Sept. to 4th Nov. 2003

Patients enrolled: 240 routine fever cases visiting for malaria test.

Type of blood sample used: Fresh whole blood directly from finger prick of fever cases. Thick and thin blood smears simultaneously prepared for microscopy.

Time of reading of test: 15 minutes after test was applied as prescribed by the manufacturer.

Gold Standard Used for Comparison: Blood smear stained with JSB stain. Blood slides blinded and read by 3 qualified Laboratory Technicians independently.

Results: Results of the testing of the kit have been summarized in Table 1 and 2 given below.

Table. 1. Comparison of malaria diagnosis with microscopy and FalciVax kit

Nos.	Microscopy	FaciVax Diag. Kit
[illegible]	[illegible]	[illegible]
Pv (% +ve)	96 (40)	96 (40)
[illegible]	[illegible]	[illegible]
Pv+Pf (% +ve)	2 (0.82)	1(0.41)

Table: 2. Shows resuits of evaluation of FalciVax malaria diagnostic Kit				
	P. falciparum (N=38)	P. vivax (N=96)	Pv+Pf (N=2)*	Overall
Sensitivity (%)	100	100	50	99.3
Specificity (%)	99.5	100	99.6	97.9
PPV (%)	97.43	100	50	98.6
NPV (%)	100	100	99.58	98.9
Efficacy (%)	99.58	100	99.17	98.8
PPV = Positive Predictive Value				
NPV = Negative Predictive Value				

*** In one case of *Pv+Pf* a band corresponding to *Pv* position on the kit appeared within 5 minutes but faded out subsequently.**

Parasitaemia: In thick blood film parasites counted against 200 WBCs to work out parasitaemia / micro litre of blood taking 8000 WBCs per micro litre as standard

Parasitaemia Range :

1. *P. falciparum*: 160 to 98400 parasites/µl of blood

2. *P. vivax* : 120- 27520 parasites /µl of blood

Inference: As table 2 reveals the kit is of good value for the diagnosis of *P. falciparum* and *P. vivax* malaria having high sensitivity, specificity and efficacy.

5/1/04

Officer - in - charge
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