Original Article

Diagnosis of Malaria Infection Using Non Radioactive Malaria Diagnostic System (NOMADS)

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Abstract

Background : Malaria remains one of the leading causes of morbidity and mortality. A definitive and early diagnosis remains the biggest challenge world-wide. Light microscopy of blood smears has been the gold standard in diagnosis of malaria for decades. This routine microscopic diagnosis is often unreliable and may not be available at many peripheral health centers. Hence newer diagnostic techniques have been developed based on antigen detection.

Method : Microscopy and Non-radioactive Malaria Detection System (NOMADS) to diagnose falciparum malaria were compared. Specificity and sensitivity of this technique and applicability of the kit for rapid and reliable malaria diagnosis were evaluated. 2579 samples of blood were processed. Both thick and thin blood smear examination and NOMADS was carried out on each of them. All smear positive samples and highly suspicious clinical cases were also subjected to detection of HRP-2 antigen by ICT Malaria Pf test.

Results : The detection rate for malaria on smear examination (both vivax and falciparum) was highest at Dimapur (7.41%), followed by Tezpur (7.13%), Kolkata (7%), Guwahati (6%) and Changsari (3.6%). All centers had greater incidence of falciparum compared to vivax except Kolkata where only vivax was detected. The sensitivity of NOMADS was 0%, 4.8%, 13.5%, 42.9% and 52.8% at Kolkata, Tezpur, Guwahati, Changsari and Dimapur respectively. The specificity of the test ranged between 91.8% at Changsari to 95.9% at Dimapur. The specificity at Tezpur, Kolkata and Guwahati was 92.3%, 94% and 95.3% respectively.

Conclusion : The study revealed that the test kit developed needs to be standardised as regards calculation of cut off values for each of the test runs and reproductibility of optical density readings. Immuno-Chromatography Test (ICT) is helpful in early diagnosis, management and follow-up of cases of malignant malaria.

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Key Words : Falciparum malaria; Immuno-Chromatography Test (ICT), NOMADS

Introduction

The definitive diagnosis of human malaria should be based on clinical criteria supported by laboratory confirmation of parasitaemia. For decades, light microscopy of blood smears has been the gold standard in diagnosis of malaria. This method depends upon the technical skill of the lab personnel. The procedure though simple demands time and patience, and ocular fatigue reduces efficiency. The microscopic diagnosis is often unreliable and may not be available at many peripheral health centers.

Newer diagnostic techniques have been developed based on antigen detection by ELISA. One of the tests is being developed on the basis of parasite specific nucleic acid sequences in the samples either by polymerase chain reaction (PCR) or by using specific complimentary biotinylated probes to detect parasite specific sequences of DNA in blood samples by nonisotopic identification method. NOMAD is one such method where biotinylated f-63 meroligonucleotideb probes are prepared synthetically and parasites in the sample are identified by way of capture hybridization [1]. This study was carried out at Command Hospital, Eastern Command [CH (EC)] to evaluate the efficacy of the kit and its utility in service hospitals. The established diagnostic method by ICT was carried out on all smear positive samples (falciparum malaria) and clinically suspicious smear negative cases to compare the results.

Material and Methods

All patients with undiagnosed fever and those with recent history of fever were included in the study. The project consisted application of Microscopy and Non-radioactive Malaria Detection System (NOMADS) to all samples for detection of *Plasmodium falciparum.* The samples were collected at five centers: CH (EC) 100 samples, 151 Base Hospital 650 samples, 155 Base Hospital 729 samples, 165 Military Hospital 850 samples and Section Hospital (Changsari) 250 samples.

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Non-radioactive Malaria Detection System (NOMADS) for Plasmodium falciparum :- The test is based on detection of a 21-base pair imperfect repeat sequence specifically present in the genome of *Plasmodium falciparum.* A DNA probe made out of this repeat sequence, which is labelled with two biotin molecules is added to lysed blood samples to hybridize with complementary parasite DNA sequences. The probe-parasite DNA hybrids are subsequently captured on microtitre plate wells, which were pre-coated with the specific nonbiotinylated DNA probe. The captured parasite DNA is detected through biotinylated probe, using a streptavidin-alkaline phosphate conjugate, which binds to biotin molecules attached to specific probe molecules. Addition of p-nitrophenyl phosphate substrate to the microtitre plate wells results in colour development if the blood sample was infected with *P.falciparum*. The manufacturer's protocol for the conduct of the test was adhered to.

ICT test :- Blood from patients collected in EDTA/Heparin was used. The test was carried out as per the recommendation of the manufacturers, ICT Diagnostics Australia. The test uses two antibodies specific for pfHRP-2 antigen. One of the antibodies is attached to visible colloidal gold impregnated into a sample pad, while the second antibody is immobilized as a line across the test strip. 10 microlitres of whole blood is added to the sample pad where lysis occurs and pfHRP2 antigen if present binds to colloidal gold-labeled antibody. When a given reagent is added to the sample pad, blood and labeled antibody migrate towards the test strip crossing the second antibody line. Appearance of a pink line indicates binding of pfHRP2 to second antibody and thus presence of *Plasmodium falciparum* antigen.

Results

A total of 2579 blood samples were collected at the five centers and both thick and thin blood smear examination and NOMADS was carried out on each of them. The established diagnostic method of detection of HRP-2 antigen by ICT Malaria Pf test was carried out in all smear positive samples (falciparum malaria) and clinically suspicious smear negative cases, to compare the results. The results of smear positivity, ICT test and NOMADS from all centers is shown in Table 1. The detection rate for malaria on smear examination (both vivax and falciparum malaria) was: Dimapur (7.41%), followed by Tezpur(7.13%), Calcutta(7%), Guwahati (6%) and Changsari (3.6%). All centers had greater incidence of falciparum compared to vivax except Calcutta where only vivax was detected.

Table 1 and 2 show comparative results of smear examination and NOMADS test from all centers. As can be seen from the comparison, NOMADS gave a wide range of sensitivity and specificity. The sensitivity of NOMADS was 0%, 4.8%, 13.5%, 42.9%, and 52.8% at Calcutta, Tezpur, Guwahati, Changsari and Dimapur respectively and specificity ranged between 91.8% at Changsari to 95.9% at Dimapur. The specificity at Tezpur, Kolkata and Guwahati was 92.3%, 94% and 95.3% respectively. The positive predictive value of NOMADS was extremely low (0-45.9%) while the negative predictive value ranged between 94.1-98.2% with 95% CI (confidence intervals) (Table 2).

Discussion

Early diagnosis of *P.falciparum* remains one of the important challenges in our country particularly in the endemic zones. Though detection of malarial parasite by microscopy remains the gold standard, the method may not detect infection in peripheral blood. It is well documented that in some cases the parasite is localized to internal organs only. A large study revealed that as

Table 1

Comparative results of smear examination. NOMADS & ICT from all centers

Centre	Samples	Smear +ve	NOMADS+ve			ICT+ve
			P vivax	P falciparum	Total	
Tezpur	729	10	41	52 (07.13)	55 (07.54)	48 (06.58)
Guwahati	650	02	37	39 (06.00)	34 (05.23)	43 (06.61)
Dimapur	850	10	53	63 (07.41)	61 (07.17)	59 (06.94)
Kolkata	100	07	00	07 (07.00)	06 (00.00)	09 (09.00)
Chattisgarh	250	02	07	09 (03.60)	23 (09.20)	18 (07.20)
All Centres	2579	31	139	170 (6.59)	179 (6.94)	177 (6.86)

(Figures in parenthesis indicate percentage of samples examined)

Table 2

Relative efficacy of NOMADS in relation to smear examination used as the Gold Standard

Smear	NOMADS					
	+	-	Sensitivity	Specificity	PPV	NPV
+ (139)	38 (TP)	101 (FN)	27.33%	94.22%	21.23%	95.79%
- (2440)	141 (FN)	2299 (TN)				
Total (2579)	179	2400				

True Positives (TP), False Positive (FP), True Negative (TN), False Negative (FN)

Sensitivity (Sen), Specificity (Spe), positive predictive value (PPV) and negative predictive value (NPV)

many as 20% pregnant ladies had placental malaria [2].

The conventional diagnosis of malaria by microscopy is laborious, requires a trained microscopist and results are subjective. In cases of outbreaks, there is a possibility of accumulation of large number of smears resulting in reduced sensitivity and increased subjectivity.

Many methods of sero-diagnosis have been developed to overcome difficulties of conventional diagnosis. These are, however, hampered by the persistence of circulating antibodies [3]. The search is for a new generation of diagnostic tests of the Parasite nucleic acid as indicative of the presence of the parasite. Experiments have shown that as few as 50 parasites/ L can be detected by nucleic acid hybridization using radioactive probes and hundreds of samples can be processed in a day [4].

DNA based diagnostic method NOMADS was used in this study to detect *P.falciparum* infection in whole blood. Many field trials have been conducted by Astra Research Foundation in endemic areas in India. Microtitre plate wells coated with varying amounts of capture probes (10-1000 ng) were used to study the sensitivity and specificity of NOMADS in diagnosis of *Plasmodium falciparum* infection[1]. The authors observed that less that 8ng of *Plasmodium falciparum* DNA(approx 2% parasitemia) was easily detected where as high content of human DNA (1 g) gave an optical density of $<0.3A_{410nm}$. They also concluded that as low as 10 ng of unlabelled f-63-mer was sufficient to capture complete hybridization [1].

This study evaluated the utility of NOMADS as compared to smear examination in detection of *Plasmodium falciparum* infection. A total of 2579 samples were processed from five centers. The detection rate for malaria (both vivax and falciparum) by microscopy was variable. It was 7.41% at Dimapur, 7.13% at Tezpur, 7% at Kolkata, 6% at Guwahati and 3.6% at Changsari. All centers had greater incidence of falciparum malaria compared to vivax except Kolkata where no falciparum malaria was detected. The NOMADS could detect 6 samples at Calcutta, 23 at Changsari, 34 at Guwahati, 55 at Tezpur and a maximum of 61 at Dimapur. Of these, all 6 were false positive at Calcutta whereas the number of false positives at Changsari, Guwahati, Dimapur and Tezpur were 20, 29, 33 and 53 respectively.

The sensitivity of NOMADS revealed an unacceptable wide range of 0% at Kolkata to 52.8% at Dimapur. The specificity of the test ranged between 91.8% at Changsari to 95.9% at Dimapur. The wide range of sensitivity and specificity was putatively due to wide variations in the optical densities and non-reproducibility of results. The principal manufacturers Astra Research Center have claimed that in their first field trial of 20 smear positive cases of *P.falciparum*, all were positive by NOMADS. In these cases the parasitaemia was >0.1%. In their field trial II out of 80 samples analyzed, 51 were positive by microscopy and 37 by NOMADS and specificity was confirmed since P vivax infected blood samples did not show any positive reaction in NOMADS. Contrary to this, the kits manufactured by the East India Pharmaceuticals with the same technical know how failed to reveal similar findings. Thus, in our study though we could achieve fairly satisfactory specificity but sensitivity of the kit is very variable and unreliable.

The PCR based tests can be used for detection of parasite sequence in samples. The major advantage is the detection of low parasitemia (as low as 5/microlitre) with 100% specificity. The sensitivity and specificity of PCR based on microscopy as gold standard are both 90%. Serological tests for malarial parasites are based on the two parasite antigens, the Histidine Rich Protein 2 (HRP-2) which is a water soluble antigen synthesized by *P.falciparum* and secreted in the blood stream and an enzyme secreted by all species of Plasmodium, i.e. Parasite Lactate Dehydrogenase (pLDH) antigen. Both antigens are secreted into blood by the asexual stage of the parasite. The pLDH is also secreted by the gametocytes. The latest antigen capture tests are rapid, simple and have detection limits comparable to those of high quality microscopy (100-200 parasites/microlitre). The serological kit used in our study was manufactured by ICT Diagnostics, Australia [5,6].

The sensitivity of ICT test was 100%. ICT test could detect all samples, which were positive for falciparum malaria by smear examination at all centers. In fact the test picked up 3 samples at Tezpur. 4 at Guwahati, 9 at Calcutta and 11 at Changsari, which were negative for falciparum malaria by smear examination. They could have been false positive. It was noticed that the disadvantage of ICT is that the antigen persisted upto 15 days after initiation of antimalarial therapy [7]. Kharakurwa et al reported a gradual decline of antigen after starting antimalarial therapy and the antigen almost disappeared by the 10^{th} day [8]. ICT was useful in the diagnosis of *Plasmodium falciparum* infection in cases of fever with shock, seizures or abnormal behaviour where parasitaemia was very low or absent, as the parasites may be sequestered in the internal organs. In such cases NOMADS was negative.

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