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Comparative Study of Sensitivity of Rapid Diagnostic (Hexagon) Test with Calculated Malarial Parasitic Density in Peripheral Blood

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ABSTRACT

Background: Different diagnostic test kits are used for rapid diagnosis of malaria. Most are based on antigen detection (pLDH, Pan Aldolase, HRP-2). In context of Nepal the diagnostic reliability and sensitivity of these tests is unknown. Hexagon Malaria Combi™ is one of the most commonly used test kit in Nepal for rapid diagnosis of malaria. The aim of the present study is to evaluate the sensitivity of the Hexagon malaria Combi test in comparison with parasitic density by microscopy technique

Methods: A Cross sectional prospective study was conducted in three districts of Nepal from September to November 2009. Blood samples were collected from the suspected cases of malaria. Thick and thin smear were prepared from all the samples and Giemsa stain was done. Simultaneously RDT (hexagon) for malaria was done. When RDT was found to be positive, blood was serially diluted in 6 tubes as 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64. RDT was done on diluted blood till RDT test gave negative result. Parasitic density was calculated for undiluted and diluted blood samples and sensitivity of RDT in various parasitic densities was calculated.

Results: Hexagon malaria combi test is sensitive (86%) when malarial parasitic density is >500/µl. Sensitivity was found to be directly related to parasitic density. Its sensitivity is very low (2.9%) when parasitic density is less than 500/ µl.

Conclusions: The sensitivity of rapid diagnostic test (hexagon Combi test detecting malarial pLDH antigen) is high only if the parasitic density is more than 500/µl.

Keywords: rapid diagnostic test, parasitic density, malarial microscopy.

INTRODUCTION

Malaria presents a diagnostic challenge to the medical community worldwide. Its occurrence is noted in more than 90 countries. It is estimated that there are more than 50 million cases and 1.1-2.2 million deaths due to malaria every year.[1] The diagnostic modalities which are available for malaria range from conventional thick and thin smear to rapid modalities like fluorescent staining (Quantitative buffy coat Technique) and antigen detection tests detecting parasitic antigens like histidine-rich protein-2 (HRP 2), plasmodium lactate dehydrogenase (pLDH) and pan-specific aldolase.[1,2] Although the peripheral blood smear examination has been the "gold standard" for the diagnosis of malaria, the Rapid diagnostic tests for the detection of malaria antigens, developed in the past decade (in 1990s), have opened a new and exciting avenue in malaria diagnosis.[3]

In Nepal (malaria endemic country), different diagnostic tests are being used for the rapid diagnosis of malaria. But the diagnostic reliability and sensitivity of these tests is not evaluated yet. So, it is necessary to evaluate the performance characteristics of these rapid test kits. Hexagon Malaria Combi™ test for the qualitative detection of pLDH released from the live parasite (all 4 human malaria species), is one of the most commonly used test kit in Nepal.[3]

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This study was conducted to compare the sensitivity of Hexagon combi test with parasitic density found in malarial microscopy and evaluate its accuracy and diagnostic capacity of minimum parasitic density that can be detected by this RDT.

METHODS

A Cross sectional prospective study was conducted in 15 places of 3 districts : Machapalan ,Chaumala Kaptan, Santoshitole, Lamki, Dhachavari, Bela, Joshipur/ Phulbari Tikapur/Lamki, Gardaria of Kailali district, Galari and Rambilaripur, Mahendranagar of Kanchanpur district, Mechi Zone, Baniyani PHC, Gauradaha, Surunga DPHO of Jhapa district. The study was also conducted in Dhanusha district but no malaria cases were detected during the study period among the study population. The study was carried out during September to November 2009.

The suspected cases of malaria (fever with chills and rigor), both male and females of all age group were included in the study. Verbal consent was taken from the patients. Cases with fever with chills, cough, and breathlessness or symptoms of Urinary tract infection were excluded. Venipuncture was done and blood was collected in EDTA vials.

Four slides were prepared, two for thick and two for thin smears, from whole blood of each patient. Thin film was fixed immediately after drying with methanol. Two slides (one thin and one thick) were stained with Giemsa at the field site and the two unstained slides were kept for transporting to NPHL.

Preliminary microscopy of smears was done to see the presence of malarial parasite and classified as negative or positive P.V./PF/Mixed infections. At least 100 HPF of the thick smears were examined at a magnification of 100X before reporting as negative. In positive cases the species of Plasmodium were identified with thin smear. The parasites were counted out of 200 WBC in thick smears of undiluted blood samples and parasitic density is calculated as the number of parasites multiplied by 40 for each microlitre of blood.[3] The parasitic density in diluted blood is determined by parasitic density in undiluted blood multiplied by dilution factor.

Hexagon Malaria Combi™ test is a rapid diagnostic test for the qualitative detection of pLDH (*Plasmodium* lactate dehydrogenase) released from *Plasmodium* species and is intended for the diagnosis of malaria.[3]

The Hexagon Malaria Combi™ test kits and blood samples were kept on a bench to equilibrate to room temperature prior to use. The test with 20 µl of whole blood was performed in strict accordance with the manufacturers' instructions. Briefly, the required quantity of conjugate strips and washing strips were inserted into different rows of the micro plate frame. One drop (30 µl) of diluent was dispensed into the conjugate wells and four drops into the washing wells. 20 µl of whole blood was added into each of the conjugate wells using disposable pipettes for each sample, mixed gently and allowed for one minute. Test strips were immersed into the conjugate wells and allowed for five minutes. Test strips were then transferred into the washing wells and allowed for 10–15 minutes for the clearance of Haemoglobin. The results were then read using the manufacturers' guide. The standard reading time was 21 minutes.[2]

If RDT was found to be positive, blood was serially diluted in 6 tubes as 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64. RDT was done on diluted blood till RDT test gave negative result. The blood diluted tubes and RDT testing strips \ were labeled accordingly. RDT positivity till last dilution is noted. The RDT tested strips were brought to NPHL for verification and comparison with parasitic density. RDT result was interpreted according to kit insert as specified in literature.

Calculation of parasitic density in undiluted and diluted blood:

Parasitic density = n x 40/µl. (From blood smear of undiluted blood)

Parasitic density according to dilution of blood

RDT positivity in dilution = dilution factor

Hence parasitic density = n x 40x dilution factor/µl

RESULTS

Out of 34 Blood samples tested for malaria by Hexagon malaria combi immunochromatographic rapid diagnostic test, sensitivity was 86% when malarial parasitic density was >500-2000/ µl and its sensitivity was very low (2.9%) when parasitic density was less than 500/ µl. Least parasitic density when RDT positive was 275/ µl for P. vivax and 564 for P. falciparum. RDT seems to be 100% sensitive above parasitic density 2000/ µl.

Table1. Demographic Profile.
Total No. of malaria cases = 34
Age group range 5 years to 52 years
Mean age = 31 years
Male (M) = 23
Female (F) = 11
M:F ratio = 2:1

Most of the cases were of age group 15-45.

Table 2. Type of Plasmodium infection, n=34.

Types of plasmodium sp.	n	%
Mixed Plasmodium falciparum and P. Vivax	1	2.9
P. falciparum	3	8.8
P. Vivax	30	88.3

Out of 34 cases tested, 30 (88.3%) were P. vivax and only 3 (8.8%) were P. falciparum and one case (2.69) with mixed infection

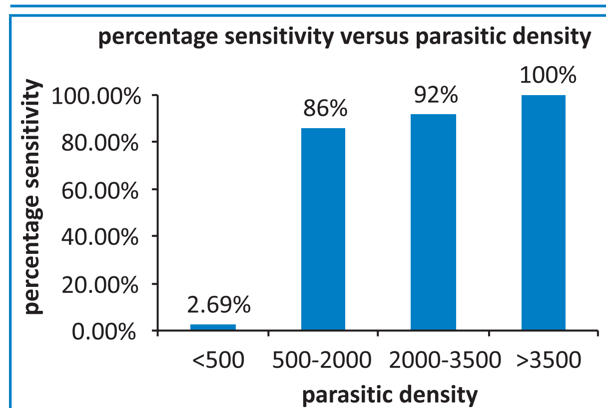

Figure 1. Relationship between parasitic density and percentage sensitivity.

Table 3. Comparison of RDT Positivity with parasitic density, n=34.

Parasitic Density	Number of Positive cases
<500/ µl	1%
>500 -2000/ µl	29%
>2000-3500/ µl	31%
>3500/ µl	34%

The test kit has very low sensitivity if parasitic density is less than 500/ µl.

Sensitivity increased with parasitic density.

Table 4. Comparison of RDT Positivity with parasitic density different species, n=34.

Parasitic Density	P. falciparum	P. vivax
<500/ µl	0%	4%
>500 -2000/ µl	100%	87%
>2000-3500/ µl	100%	100%
>3500/ µl	100%	100%

Table 4 shows that sensitivity for P. falciparum below parasitic density 500/ µl is almost zero. But It is found to be 100% sensitive in both cases if parasitic density is more than 2000/µl

DISCUSSION

Rapid detection and effective treatment is a pre-requisite for reducing the morbidity and mortality due to malaria. Microscopy of Leishman or Giemsa stained thick smears are considered to be the 'Gold standard' in diagnosis.[1] However, the interpretation of thick smears is laborious and results depend on the quality of microscope, staining, technique with which blood film is prepared and also the concentration, motivation and skill of microscopist.[1,2,5] The recent development of rapid and accurate tests for the detection of malaria is highly commendable.[2] One of the major goals of developing such rapid tests was that these rapid tests should be handled with ease and accurately by relatively unskilled staff for prompt diagnosis of malaria.[2,4,6]

Many studies have achieved >95% sensitivity at parasitemia of ~500 parasites/µL, but this high parasitemia is seen in only a minority of patients. The specificity appears to be better with the pLDH test than the PfHRP2/PMA test for both P. falciparum and non-falciparum malaria. The expected species sensitivities are 90% for *P. falciparum*, 89% for *P. vivax* while the specificity of the test is 99.5%.[1,2,3,6] The sensitivity of the RDTs at low levels of parasitemia and for non-immune population remains a problem.[3,4] The 1999 WHO Expert Committee on Malaria (2000) recommended a 95% sensitivity at 100 parasites/ µl as a target for RDT performance.

This study was aimed to evaluate the diagnostic capacity of Rapid diagnostic test kits (Hexagon malaria combi, antigen detection) in comparison of traditional microscopy. Total 34 cases were found to be positive by both RDT and Microscopy method. Out of total 34 positive cases, 30 (88.3%) were P. vivax and only 3 (8.8%) were P. falciparum and one case (2.69) with mixed infection.

Each sample was serially diluted in order to obtain the low parasitic density. All the samples gave positive RDT result above the parasitic density >3500/µl, (i.e. sensitivity of RDT was 100%), 29 out of 34 (86%) samples gave positive results within parasitic density 500-2000/ µl. Only one sample is found to be positive in parasitic density lower than 500/µl. This showed that at the low parasitic density, Hexagon malarial Combi test is almost insensitive.

A similar study conducted in Cameroon showed that when compared with microscopy as the gold standard, 64 out of the 75 samples positive by microscopy were positive by the rapid test giving a sensitivity of 85.33%. A total of 186 asymptomatic children were tested; blood films identified 75 (40.32%) of these as positives while the rapid test identified 64 (34.41%). The sensitivity and specificity of the rapid test increased as parasite density increased and performance characteristics dropped as parasite densities decreased.[2]

This study gave similar results to the study conducted in India which showed that 82 samples were positive by thick smear and 62 samples were positive by the antigen detection test (76%).[1] Similar results were obtained by a study conducted in Tanzania; with overall sensitivity of 90.7%.[7] A similar preliminary study in Madagascar in 2003 showed that rapid test detecting pLDH is highly sensitive indicator of P. falciparum infection with parasitemia exceeding 500 trophozoites/µl (sensitivity of 97.2%).[8,9,10]

The possible explanations for discrepancies between rapid diagnostic test and microscopy may include: (i) insufficient detection of low parasitaemia by the rapid test (ii) possible genetic heterogeneity of PfHRP2 expression, deletion of HRP-2 gene, presence of blocking antibodies for PfHRP2 antigen or immune-complex formation, prozone phenomenon at high antigenemia or to unknown causes.[2,11,12,14] (iii) Sequestration of parasites and (iv) false-negative reactions (v) Hexagon Malaria Combi™ rapid test detects only live parasites producing pLDH.[2,15,16]

Despites of some limitations, like low sample size, few positive cases and fewer cases with low parasitic density, this study indicated clearly that, Hexagon malarial Combi test is sensitive enough to detect malarial cases with high parasitic density only.

CONCLUSIONS

The detection capacity or sensitivity of rapid diagnostic test (hexagon Combi test detecting malarial pLDH antigen) is high only if the parasitic density is more than 500/µl. Hence microscopy technique will remain gold standard for detecting malaria when parasitic density is low i.e. less than 500/µl.

The conclusion of this study is based on test done on 34 positive cases only. In future, it is recommended to carry out the study in larger population in the month between June to August, when number of malaria cases will be high.

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