The sensitivity and specificity of a urine based Rapid Diagnostic Test for the diagnosis of *Plasmodium falciparum* in a malaria endemic area in Odisha, India

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ABSTRACT

Background: Rapid and accurate diagnosis is crucial in the treatment of malaria. Rapid Diagnostic Tests (RDTs) using blood have been recommended by the WHO as an acceptable method for the diagnosis of malaria. RDTs provide results quickly, is simple to use and easy to interpret. However, its use requires collection of blood by skin puncture. Hence the aim of the pilot study is to explore the sensitivity and specificity of RDTs using urine (collected non-invasively) for diagnosis of *Plasmodium falciparum* malaria and to assess the relation between parasite density in blood with HRP-2 Ag detection in urine. **Material and Method:** All fever cases admitted to Ispat General Hospital (IGH) Rourkela, India, during June 2012–March 2013 with a clinical diagnosis of malaria were examined for the presence of asexual forms of *P. falciparum* in peripheral blood smears. All smear positive febrile patients who met the eligibility criteria were enrolled. Smear negative fever cases were enrolled as control cases. RDTs were performed using both urine and blood samples by using commercially available blood specific kits. **Results:** Sixty blood smear positive cases and 51 febrile blood smear negative cases were enrolled. Sensitivity and specificity of RDT urine were 86.67% (95%CI:75.83–93.09) and 94.12% (95%CI:84.08–97.98) respectively whereas those of RDT blood were 91.67% (95% CI: 81.93–96.39) and 98.04% (95% CI 89.7–99.65). The sensitivity of both RDT urine as well as RDT blood were found to be dependent on the level of parasitemia. **Conclusion:** Results of this study are promising. Larger studies are needed to assess whether RDTs using urine could serve as a practical, reliable method for the detection of *P. falciparum* in a non-invasive manner where invasive blood taking is less feasible.

KEYWORDS

Malaria; diagnosis; Rapid Diagnostic Test; urine; blood

Introduction

Malaria continues to be a major cause of morbidity and mortality in developing countries. Both under and over diagnosis of malaria add a further challenge to the medical community, due to non-availability of reliable, affordable, easy and prompt malaria diagnostic methods. The accepted gold standard for diagnosis of malaria is demonstration of the *Plasmodium* parasite in thick and/or thin blood smears [1]. Unfortunately there are certain obstacles in getting a quality microscopic test results due to improper staining of blood film, inexperienced technicians, or low parasitemia. Detection of malaria using a Real-Time Polymerase chain reaction (RT-PCR) is 50 times more sensitive than microscopy but it requires costly equipment, high level of technical expertise and more time and is therefore unsuitable for clinical use on a large scale [2]. There was a search for easy, rapid and reliable test kit. The development of the Rapid Diagnostic Tests (RDTs), which are antigen capture based test, enable the rapid and accurate diagnosis of malaria.

Most RDTs used for the detection of *Plasmodium falciparum* detect the Histidine Rich Protein (HRP-2). This protein is water soluble and produced by asexual and early sexual forms of *P. falciparum* [3].

RDTs using blood (RDT-B) have been recommended by the WHO as an acceptable method for parasitological diagnosis of malaria. In order to standardize the sensitivity of the malaria RDT kits the WHO has set the lowest level of detection to 200 parasites/ μ l of blood [4]. RDTs using blood can provide results quickly, are simple to use and easy to interpret [1]. However, its use requires collection of blood by lancet/needle puncture which carries some risk of injury and disease transmission. In addition, difficulties could arise in communities where patients are reluctant to have blood drawn or that are located in remote areas. These factors could affect the feasibility of for instance large scale epidemiological studies.

As urine is an ultra-filtrate of blood and the HRP-2 antigen is a water-soluble protein, it could potentially be detected in urine. Therefore urine could be used as an alternative body fluid for the detection of *Pf* malaria.

Hence the aim of the study is to explore the sensitivity and specificity of RDT using urine for diagnosis of *P. falciparum* malaria by comparing with gold standard blood smear microscopy and to assess the correlation between parasite density in blood and HRP-2 Ag detection in urine.

Material and method

Study site

The study was carried out at Ispat General Hospital (IGH), Rourkela, situated in Sundergarh district of Odisha, India. Odisha has a population of about 42 million (3.5% of India total population). In 2010 Odisha contributed 24% of the total number of malaria cases and 17% of the total malaria mortality in India [5]. Sundergarh district has a population of approximately 2.5 million and is an endemic zone for malaria. Although transmission occurs throughout the year, high transmission occurs from July to November (monsoon season). Rourkela is an industrial city with a population of approximately 1 million. IGH is a 685-bedded tertiary care hospital with a large catchment area beyond Odisha, including Jharkhand, Bihar, Madhya Pradesh, Chattisgarh. This catchment area consists of plains, forested and mountainous areas, mines, urban, semi-urban and rural areas. The slide positivity rate for *Pf* malaria at Ispat General Hospital is 4% (Unpublished hospital data; 2011).

Study design and enrolment criteria

This prospective observational case control study was conducted between June 2012 and March 2013, after getting clearance from the Institutional Ethical Committee. All fever cases of both genders >5 years admitted to IGH during this period with a clinical diagnosis of malaria were consecutively examined for the presence of asexual forms of *P. falciparum* in a peripheral blood smear. Smear positive menstruating and pregnant women, patients with renal disease and rheumatoid arthritis were excluded from the study, as both proteinuria as well as hematuria could influence the performance of the test. Smear positive febrile patients who met the eligibility criteria were enrolled. A total of 51 smear negative (non-malarial) febrile patients were enrolled as control cases. Written informed consent was obtained from all the subjects before enrolment.

Sample collection and procedure

Thick and thin blood films were prepared from blood obtained through a finger prick in all febrile patients admitted to the hospital with clinical diagnosis of malaria. The smears were stained with Giemsa stain according to Guidelines of WHO and microscopically examined by two experienced microscopists for the

presence of asexual form of *P. falciparum* [6]. Microscopy was considered positive only if asexual parasite forms (trophozoites and schizonts) were detected. A blood film was considered negative when examination of 100 thick film fields did not show presence of asexual form of *P. falciparum*. Discordant results between the two microscopists in diagnosis and species identification were excluded from the study. 2 ml of venous blood in an EDTA tube and 1 ml of urine in a sterile tube were collected from all individuals enrolled in the study. Parasitemia in all malaria positive cases was quantified per 200 WBC from thick smear. Parasitemia per μL of blood was calculated using the following formula: Total no. of parasites/ μL of blood = Parasite count/200 WBCs $\times 8000$. This calculation assumes a WBC count of 8000/ μL of blood [1,6].

RDTs were performed using both urine (RDT-U) and blood (RDT-B) samples of the same patient by using commercially available kits (BinaxNOW Malaria Test kit, Inverness Medical, Europe). The procedure was done as per manufacturer instructions by a technician who had no knowledge of the microscopy test results. A sample of 15 μL of blood or urine was added in the sample well and allowed to flow along the test stick. In the case of the blood sample 1-2 drops of buffer was added. If both control and test bands appeared this was interpreted as positive. Appearance of only the control band was interpreted as negative. All fever cases diagnosed as malaria with a positive slide microscopy or a positive RDT (blood) were treated promptly as per WHO treatment guidelines, 3rd edition, 2015. No treatment was given based on RDT urine results. All 60 smear positive cases (study cases) and one RDT blood positive but smear negative (control cases) were treated with anti-malarial drugs.

Statistical analysis

Sample size justification

Approximate population size of our city is 1,000,000. Hypothesized % frequency of outcome factor (*Pf* malaria) in the population p , as per our unpublished hospital data 2011 = $(4\% \pm 0.5)$. confidence limit as % of 100 (absolute $\pm\%$) $d = 5\%$. design effect for cluster survey $\text{diff} = 1$. sample size, n for 95% confidence level = 59. So, our sample size should be ≥ 59 .

Data analysis

Data was analyzed using Open Epi, version 3.01 (2013) a free statistical software developed by Emory University, USA. The sensitivity and specificity were calculated by comparing RDT results of urine and blood against microscopy. The results of the RDT-U and RDT-B tests were correlated with parasite density at different levels of parasitemia.

Results

A total of 381 fever cases with the clinical diagnosis of malaria were screened through a slide microscopy. Out of these 62 were blood smear positive for *P. falciparum* malaria. Two cases were excluded based on preexisting renal disease. Sixty cases (59 with uncomplicated malaria and one case of complicated malaria) were enrolled in the study. Fifty-one febrile blood smear negative individuals were enrolled as control cases.

Out of the 60 microscopy positive cases 52 (86.67%) were RDT-U positive (Table 1) and 55 (91.67%) were RDT-B positive (Table 2). Five out of 8 negative RDT-U samples were also RDT-B negative. Out of 51 microscopy negative febrile controls 3 (5.88%) were RDT-U positive (Table 1) and 1 (1.96%) was RDT-B positive (Table 2). There was no overlap in RDT-U and RDT-B positive cases in slide negative fever cases.

In this study sensitivity and specificity of RDT-U were 86.67% (95%CI: 75.83–93.09) and 94.12% (95% CI: 84.08–97.98) respectively whereas those of RDT-B were 91.67% (95%CI: 81.93–96.39) and 98.04% (95%CI 89.7–99.65).

The sensitivity of both RDT-U as well as RDT-B were found to be dependent on the parasitemia found through examination of the thick blood smear (Tables 3 and 4). In cases with a parasitemia of <200 parasites/ μ L the RDT-U was negative in 5 out of 5 cases whereas the RDT-B was found to be negative in 4 out of 5 cases. In the 8 cases with a parasitemia between 200 and 500 parasites/ μ L the number of false negative results was 3 versus 1 using the RDT-U and RDT-B methods, respectively. No false negative RDT-U or RDT-B results were observed in the 47 cases with a parasite density >500 parasites/ μ L of blood.

Discussion

Rapid and accurate diagnosis is crucial in the treatment of malaria as delays in diagnosis lead to a delayed treatment which is associated with higher morbidity and mortality. Currently, WHO has recommended parasitological confirmation of suspected malaria cases prior to treatment [7]. The reading of blood smears and detection of malaria DNA through PCR are highly sensitive and specific methods. However, they are time consuming, costly and require adequately trained staff and adequate facilities. Rapid Diagnostic Tests, which can be performed using blood obtained through a finger prick, provide rapid results and can be performed in resource limited settings without the need for much training. Although the blood based RDTs are well accepted throughout the world as a diagnostic tool their disadvantage is the need for the collection of blood. As urine is non-invasively collected, the possible importance of urine test is more in situations when blood collection is difficult or unacceptable. In this pilot study, we assessed the sensitivity and specificity of RDTs (developed for detecting PfHRP-2 in

Table 1. Comparison of results of RDT urine vs. blood Microscopy.

RDT (urine)	Microscopy		
	Positive	Negative	Total
Positive	52	3	55
Negative	8	48	56
Total	60	51	111

Table 2. Results of RDT blood vs. blood microscopy.

RDT(blood)	Microscopy		
	Positive	Negative	Total
Positive	55	1	56
Negative	5	50	55
Total	60	51	111

Table 3. Correlation between parasite density and RDT urine.

Parasite count / μ L of blood	Number of cases by RDT urine		
	Positive	Negative	Total
<200	0	5	5
200–500	5	3	8
501–1000	7	0	7
1001–2000	3	0	3
>2000	37	0	37
Total	52 (86.67%)	8 (13.33%)	60 (100%)

Table 4. Correlation between parasite density and RDT blood.

Parasite count/ μ L of blood	Number of cases by RDT blood		
	Positive	Negative	Total
<200	1	4	5
200–500	7	1	8
501–1000	7	0	7
1001–2000	3	0	3
>2000	37	0	37
Total	55 (91.67%)	5 (8.33%)	60 (100%)

blood) for the diagnosis of *P. falciparum* malaria through the use of the urine of febrile patients admitted in our hospital.

This study indicates that the sensitivity and specificity of RDT-U and RDT-B are comparable. However, it is possible that this study was underpowered to detect a significant difference between the two tests. The sensitivity of both RDT-U and RDT-B depended on the parasitemia found through microscopy, with negative test results in 5 (RDT-U) and 4 (RDT-B) out of 5 cases with a parasitemia count below 200 parasites/ μ L. Sensitivity of both test increased as the parasitemia increased with detection of all slide positive cases with a parasite count >500/ μ L by both RDT-U and RDT-B. At higher parasite density ($\geq$ 200 parasites/ μ L of blood), the sensitivity and specificity of both RDT-U and RDT-B were comparable [1,3].

The use of body secretions that can be obtained in a non-invasive manner, such as saliva and urine, for the diagnosis of malaria by detection of parasite antigens and/or DNA has been suggested and explored several times before [4,7–14].

In 1991 Rodriguez et al. detected *P. falciparum* specific antigens and antibodies by immune-fluorescence in the urine of 46 persons in Thailand and Ghana [12].

In a study in 1998 involving 126 children in Papua New Guinea the ParaSight(R)-F, an antigen based test using blood, was found to have a sensitivity and specificity of 84 and 77% respectively versus 81% and 26% when applied to urine [13]. The authors noted that the amount of malaria antigen depends on the time of collection of sample, suggesting that using the morning urine might improve sensitivity.

Wilson et al (2008) found an ELISA positivity for HRP-II in 16 (53%) plasma samples versus 14 (47%) saliva samples obtained from 30 children with a positive blood smear, with no false positivity using both tests [14].

In 2014, Oguonu et al evaluated the performance of urine malaria test (HRP-II antigen based) in 195 febrile subjects and found sensitivity and specificity of 83.75 and 83.48%, respectively [4].

In 2017, Oyibo et al evaluated Urine Malaria Test (UMT) for *P. falciparum* malaria diagnosis in febrile patients and found sensitivity to be 85% and specificity to be 84% respectively [5].

Potential factors that could influence the sensitivity of HRP-2 based RDTs using urine are the level of parasitemia, antigen production by the parasite (which for instance could be influenced by mutations leading to lower HRP-2 antigen production), rate of antigen filtration (which could depend on the time of day), presence of antibodies to HRP-2 Ag and the properties of the antibodies (monoclonal and/or polyclonal) that are impregnated on the RDT kits [3,5,15,16]. Potentially sequestration of parasites could limit antigenemia and subsequent ultrafiltration of these antigens to the urine [5].

Finally, degradation or proteolytic cleavage of urine excreted proteins could affect the performance of RDTs that are developed for the detection of the intact antigen in blood [8].

The false positive results observed in this study could be due to the presence of gametocytes, persistence of the antigen for 3–4 weeks after a previous malaria infection in a high transmission area, and cross reactivity of the RDT with other antigens [4]. In this study the specificity of both RDT-U and RDT-B was comparable. The 3 false positive cases detected by RDT-U were patients with septicemia (2) and Acute Respiratory Distress Syndrome (ARDS). Both conditions are known to be accompanied by a noticeable acute phase protein increase which might have contributed to these false positive results.

The use of blood based RDTs might have affected the performance of the RDT-U in this study. However, the results indicate nearly equal performance of both RDT-U and RDT-B which could indicate that the kit used in this study could be of value for the noninvasive detection of *P. falciparum* malaria.

In this study, we have used the presence of *P. falciparum* in blood films as the reference for both the

RDT-U and RDT-B. Potentially the use of PCR could have improved the accuracy of the initial detection of *P. falciparum* infections. However, the use of PCR could lead to the detection of subclinical infections that might not be of clinical significance. The exclusion of pregnant or menstruating women, patients with renal disease and/or rheumatoid arthritis prevents generalizing these results to the whole population.

Conclusion

In this study the sensitivity and specificity of RDT-U and RDT-B were comparable, with the sensitivity of both methods correlating with the level of parasitemia. The results of this study are promising. However, larger studies are needed to assess whether RDTs using urine could serve as a practical, reliable method for the detection of *P. falciparum* in a non-invasive manner where invasive blood taking is less feasible.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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