Comparative Evaluation of Bivalent Malaria Rapid Diagnostic Tests versus Traditional Methods in Field with Special Reference to Heat Stability Testing in Central India

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Abstract

Background: Malaria presents a diagnostic challenge in areas where both Plasmodium falciparum and P.vivax are co-endemic. Bivalent Rapid Diagnostic tests (RDTs) showed promise as diagnostic tools for P.falciparum and P.vivax. To assist national malaria control programme in the selection of RDTs, commercially available seven malaria RDTs were evaluated in terms of their performance with special reference to heat stability.

Methodology/Principal Findings: This study was undertaken in four forested districts of central India (July, 2011– March, 2012). All RDTs were tested simultaneously in field along with microscopy as gold standard. These RDTs were stored in their original packing at 25°C before transport to the field or they were stored at 35°C and 45°C upto 100 days for testing the performance of RDTs at high temperature. In all 2841 patients with fever were screened for malaria of which 26% were positive for P.falciparum, and 17% for P.vivax. The highest sensitivity of any RDT for P.falciparum was 98% (95% CI; 95.9–98.8) and lowest sensitivity was 76% (95% CI; 71.7–79.6). For P.vivax highest and lowest sensitivity for any RDT was 80% (95% CI; 94.9 - 83.9) and 20% (95% CI; 15.6–24.5) respectively. Heat stability experiments showed that most RDTs for P.falciparum showed high sensitivity at 45°C upto 90 days. While for P.vivax only two RDTs maintained good sensitivity upto day 90 when compared with RDTs kept at room temperature. Agreement between observers was excellent for positive and negative readings for both P.falciparum and P.vivax (Kappa >0.6–0.9).

Conclusion: This is first field evaluation of RDTs regarding their temperature stability. Although RDTs are useful as diagnostic tool for P.falciparum and P.vivax even at high temperature, the quality of RDTs should be regulated and monitored more closely.

Introduction

Malaria due to both Plasmodium falciparum and P. vivax is a life threatening disease for individual with low immunity [1], [2]. However, it is usually curable if diagnosed quickly [3], [4], [5]. The importance of obtaining results quickly from the examination of blood samples from suspected malaria patients is now made possible with the introduction of Rapid Diagnostic Tests (RDTs). Malaria RDTs were introduced in the nineties and have undergone many improvements [6], [7], [8]. The number of RDTs available, and the scale of their use has rapidly increased over the past few years [9], [10]. By now more than 60 RDT brands and over 200 different products have been developed [11] and the number of malaria RDTs produced annually has increased from 45 million in 2008 to 88 million in 2010 [12]. RDTs are hand held cassettes detecting Plasmodium parasites by an antibody antigen reaction [13]. These RDTs are available in several formats (lateral flow cassette, dipstick & cards etc) detecting one or more antigens (HRP-2 or pLDH or Aldolase or in combination). Although RDTs showed promise as new diagnostic tools, it is not clear which RDT is most appropriate for different epidemiological settings where both P. vivax and P. falciparum are co-endemic. Presumptive treatment of all fevers as malaria with chloroquine (CQ) becomes increasingly popular in resource poor setting because of lack of laboratory infrastructure and technical expertise [14], [15]. In such settings, empirical treatment results in substantial overuse of anti-malarial drugs and delays the diagnosis of other febrile illness [16], [17].
Malaria RDTs have the potential to provide a huge step forward in the management of febrile illness in malaria-endemic areas [12]. However, declining sensitivity of RDTs from field was also reported [18], [19]. There are several possible reasons for this i.e. HRP-2 gene deletions [20], [21] exposure to high temperature with or with out high humidity [22], operational difficulties and human error. High humidity accelerates denaturation [12], [23]. Therefore, to assist national malaria control programmes and other procurement agencies in the selection of products appropriate to their needs, commercially available bivalent malaria RDTs were evaluated in terms of their performance with special reference to stability testing in forest villages of Central India (Madhya Pradesh).

Materials and Methods

This study was carried out in tribal and forested areas of four malarious districts of Madhya Pradesh i.e. Jabalpur, Mandla, Balaghat and Rewa during July 2011 to March 2012 (Figure 1). A cross sectional study design was used for assessing the performance of RDTs. Bivalent commercially available RDTs were selected for this study. The seven RDTs used in this study are – FIRST RESPONSE® malaria antigen pLDH/HRP2 combo card test (Premier medical corporation Ltd, Daman), GENOMIX/Pf/Pv) Malaria Antigen Detection test cassette (GENOMIX Molecular Diagnostics Pvt. Ltd., Hyderabad, Andhra Pradesh). Falcivax Rapid test for malaria Pf/Pf Device (Zephyr Biomedical, Verna, Goa), Parascreet®, Rapid test for malaria Pan/Pf Device (Zephyr Biomedicals, Verna, Goa), ParaHIT® Total Pan/Pf (Span Diagnostics Ltd., Surat, Gujarat), SD BIOLINE malaria Ag Pf/ Pan (SD Bio Standard Diagnostics Pvt Ltd., Gurgaon, Haryana) and NecVIPARUM one step malaria Pf/Pv antigen detection test (Nectar Life Science Ltd., Chandigarh). These RDTs were procured directly from the manufacturers or their authorized dealers and stored in their original packing at 25°C before transport to the field sites (Table S1).

A one week training workshop was organized in the month of July 2011 to train the project staff for standard procedure of data collection and result interpretation. This was followed by two days training in field for screening and enrollment of study subjects, taking consent, collection of blood samples and RDT test procedures. The field clinic traveled 10 villages of each district for screening patients. All households of selected villages were visited by survey team and all clinically suspected malaria cases (as per National Programme guideline) were enrolled for the study. There were two teams, each consisting of two field workers, one technician and one research assistant.

All 7 RDTs were tested simultaneously in field on all enrolled patients using whole blood collected by a finger prick in a single bleed after taking informed consent. RDTs were performed at each site by members of the study team i.e. field worker and technician as per manufacturers instruction, and results interpreted and recorded after 15 to 30 minutes. They were advised that if the background of the RDT test window remained pink after 15 min, they should wait till the background is clear before declaring the test results. The study team recorded each RDT result as either positive or negative or invalid. Thick and thin smears were prepared from the same finger prick blood and air-dried. At the central laboratory of Regional Medical Research Centre for Tribals, thick and thin smears were stained with JSB [24] and examined by microscopist who was unaware of the RDT results. The microscopist examined 100 microscopic field of thick smear before declaring a smear as negative. When results of the RDT and microscopy were discrepant, smears were reviewed by a second independent microscopist unaware of previous result. Parasite densities were calculated according to the standard method (parasite/μl = no. of asexual parasites × 3800/no. of WBC counted). All positive and 10% negative blood smears and all the discordant results on presence or absence of parasitaemia between the two microscopists were resolved by referring to a third expert microscopist. These microscopists, examining blood smears were blinded to result of RDT, clinical status of patients and the microscopy.

Ethics Statement

This study was approved by institutional review board of Regional Medical Research Centre for Tribals, Jabalpur India (IRB00006471). Written informed consent were obtained from all participants or the parents of children younger than 18 years, and assent were taken from children of age between 7 to <18 years as per Ethical Guidelines of Indian Council of Medical Research, New Delhi India.

Heat Stability Testing

For temperature stability testing of the RDTs which were stored at 25°C on receipt, were then allocated to separate groups for storage at 35°C & 45°C up to 100 days and at 60°C for 48 hrs. The incubators were stabilized at the required temperature for three days before the RDTs were placed inside. The log book was maintained for these incubators and temperature was monitored three times in a day. RDTs were removed from storage at 15 to 30 days time intervals allowed to reach at room temperature before testing. For all temperature/time combinations studied seven RDTs were tested for each temperature/time point and comparison was made with control RDTs (one for each temperature/time point). A micropipette was used to measure blood volume. The reading of the tests was undertaken by the same person to minimize inter-operator variability. The temperature during transport and in field were not monitored as it was not controlled.

Sample Size

We assumed that the sensitivity of RDT is approximately 90% (P = 0.90) and absolute precision of 5% (d = 0.05), i.e. the estimated sensitivity of RDT will vary between 85-95%. The minimum required number of cases (positive cases by gold standard) is 138. Our past experiences in the study area revealed that P. falciparum is more prevalent than P. vivax in most parts of Madhya Pradesh. Thus, assuming slide falciparum rate (SFR) as 25% and slide vivax rate (SVR) as 15% for the study area, we need to screen about 552 cases for P. falciparum and 920 cases for P. vivax. Thus, overall we need to screen about 1500 malaria suspected cases to test the sensitivity of different RDTs for both P. falciparum and P. vivax.

For heat stability testing, we assumed that the sensitivity of RDT kit is approximately 90% (P = 0.90) and absolute precision of 10% (d = 0.1), i.e. the estimated sensitivity of RDT kit will vary between 80-100%. The minimum required number of cases (positive cases by gold standard) is 35 at each level of temperature.

Treatment

All patients infected with P. falciparum and P. vivax were given treatment as per treatment guideline of National Vector Borne Disease Control Programme (NVBDCP) [25]. All adult subjects with P. falciparum were administered the oral dose of ACT (1500 mg Sulfadoxine, 75 mg Pyrimethamine and 600 mg of Artesunate divided into 3 days) with single dose of Primaquine (45 mg). P. vivax cases were given 1,500 mg Chloroquine for three
days, followed by 15 mg Primaquine daily for 14 days. Infants and
children were given proportionally lower doses. Infants and
pregnant women were not given Primaquine.

Data Entry and Analysis

The forms were double-entered using CS-Pro 4.1 (US Census
Bureau, Washington, DG, USA), with range, consistency, and edit
checks built into the data entry programme for quality control.
The two databases were validated and all inconsistencies and
differences were resolved. Statistical analyses were performed
using STATA 11.2 (StataCorp Texas USA). Diagnostic perfor-
ance characteristics i.e. sensitivity, specificity and positive &
negative predictive values (PPV & NPV) were calculated against
light microscopy as gold standard by using ‘diagt’ command in
STATA. However for heat stability testing sensitivity and
specificity were calculated against diagnostic performance of
RDTs kept at room temperature.

During calculation of sensitivity/specificity, matching mixed
infections with *P. falciparum* and *P. vivax* were taken as true positive
for both *P. falciparum* and *P. vivax* species and *P. falciparum* only
gametocyte cases were considered as true negative. *P. malariae*
infections were excluded during the analysis of RDTs perfor-
mance. Inter-observer agreement for both results of positive and
negative reading as well as for stability was expressed by Kappa
statistics for each pair of observers. A Kappa between 0.6 and 0.8
was considered a good agreement, higher than 0.8 was considered
as excellent [26].

Results

In all 2841 patients aged 2 month to 80 years (median age 10
years) with fever/history of fever were screened for malaria. Only
2207 eligible subjects were enrolled for RDT testing in parallel
with microscopy. The enrollment of study subjects are divided into
two parts i.e. one for testing RDT diagnostic performance under
normal field condition and another for heat stability testing by
keeping RDTs at various temperature up to 100 days.

Study Part I

In this part of the study, 1807 subjects were tested by all 7 RDTs
in field. Of which 46.1% were positive, 25.7% *P. falciparum*, 16.6%
*P. vivax*, 1.0% *P. malariae*, 1.9% mixed infection of *P. falciparum* and
*P. vivax* and 0.9% mixed infection of *P. malariae* with *P. falciparum
and/or P. vivax*. FIRST RESPONSE detected 468 out of 480
microscopically confirmed asexual & sexual falciparum malaria
infection (Table 1). However, other RDTs i.e. FalciVax detected
429, parascreen 425, SD BIOLINE & NecVIPARUM 416, ParaHIT Total 373 and GENOMIX detected only 360
falciparum infections (Table 1). Among microscopically confirmed
329 *P. vivax* subjects, FIRST RESPONSE detected 262,
parascreen 162, SD BIOLINE 163, FalciVax 149, NecVIPARUM 141, ParaHIT Total 112, while only 65 cases were
detected by GENOMIX (Table 2). Number of invalid test
(absence of control band) was recorded in 1, 7, 2, 8, 9, 15 and 4
tests respectively for FIRST RESPONSE, parascreen, Para-
HIT Total, FalciVax, SD BIOLINE, GENOMIX and NecVIPARUM.
The analysis of results revealed that the sensitivity of the FIRST RESPONSE® for *P. falciparum* was 98% (Table 1), of parascreen® and FalciVax 89%, SD BIOLINE & NecVIPARUM 87% and ParaHIT® Total 78%, whereas the sensitivity of GENOMIX was only 76%. The specificity for *P. falciparum* was 92% by GENOMIX, 91% by ParaHIT® Total, 90% by FIRST RESPONSE® and NecVIPARUM, 86% by parascreen®, 85% by SD BIOLINE and 84% by FalciVax.

For *P. vivax*, the sensitivity of different tests when compared with microscopy were 80% by FIRST RESPONSE®, 50% by parascreen® and SD BIOLINE, 46% by FalciVax, 43% by NecVIPARUM and 34% by ParaHIT® Total, while only 20% by GENOMIX (Table 2). Specificity of all these tests ranged between 97–99%.

Analysis of sensitivity on different level of parasitaemia revealed that FIRST RESPONSE® was able to detect 100% malaria infection at >100 parasites/µl of blood for both *P. falciparum* and *P. vivax*. While parascreen®, FalciVax, SD BIOLINE and NecVIPARUM were able to detect >90% *P. falciparum* infections when parasite densities were >500 parasites/µl. However, ParaHIT® Total and GENOMIX detects 90% *P. falciparum* infections when parasitaemia was >1000 parasites/µl. Regarding *P. vivax* infections except FIRST RESPONSE®, other RDTs detect only 31–63% when parasite density is >500 parasites/µl (Figure 2).

### Table 1. Evaluation of *P. falciparum* by seven different RDTs against light microscopy as gold standard in four district of Madhya Pradesh, central India.

|                     | FIRST RESPONSE® | Parascreen® | ParaHIT® Total | FalciVax | SD BIOLINE | GENOMIX | NecVIPARUM |
|---------------------|-----------------|-------------|----------------|----------|------------|----------|------------|
| Sensitivity (95% CI) | 97.7 (95.9–98.8) | 88.9 (85.7–91.6) | 77.9 (73.9–81.5) | 89.4 (86.3–92.0) | 86.8 (83.5–89.7) | 75.8 (71.7–79.6) | 86.7 (83.3–89.6) |
| Specificity (95% CI) | 90.1 (88.4–91.7) | 85.8 (83.8–87.6) | 90.6 (88.9–92.1) | 84.1 (82.0–86.0) | 85.4 (83.3–87.2) | 91.9 (90.3–93.3) | 89.6 (87.9–91.2) |
| PPV (95% CI)          | 78.1 (74.6–81.4) | 69.3 (65.5–73.0) | 74.9 (70.8–78.7) | 67.1 (63.3–70.8) | 68.3 (64.4–72.0) | 77.1 (73.0–80.8) | 75.2 (71.4–78.8) |
| NPV (95% CI)          | 99.1 (98.4–99.5) | 95.5 (94.2–96.6) | 91.9 (90.3–93.3) | 95.6 (94.3–96.7) | 94.7 (93.3–95.9) | 91.3 (89.7–92.8) | 94.9 (93.5–96.0) |

TP 468 425 373 429 416 360 416
TN 1196 1134 1201 1109 1126 1210 1186
FP 131 188 125 210 193 107 137
FN 11 53 106 51 63 115 64

NPV (95% CI) 99.1 (98.4–99.5) 95.5 (94.2–96.6) 91.9 (90.3–93.3) 95.6 (94.3–96.7) 94.7 (93.3–95.9) 91.3 (89.7–92.8) 94.9 (93.5–96.0)
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Sensitivity (95% CI) 79.6 (74.9–83.9) 49.5 (44.0–55.1) 34.3 (29.1–39.7) 45.7 (40.2–51.3) 49.5 (44.0–55.1) 19.8 (15.6–24.5) 42.9 (37.4–48.4) 843.8 (23.5–86.0)

Table 2. Evaluation of *P. vivax* by seven different RDTs against light microscopy as gold standard in four district of Madhya Pradesh, central India.

|                     | FIRST RESPONSE® | Parascreen® | ParaHIT® Total | FalciVax | SD BIOLINE | GENOMIX | NecVIPARUM |
|---------------------|-----------------|-------------|----------------|----------|------------|----------|------------|
| Sensitivity (95% CI) | 79.6 (74.9–83.9) | 49.5 (44.0–55.1) | 34.3 (29.1–39.7) | 45.7 (40.2–51.3) | 49.5 (44.0–55.1) | 19.8 (15.6–24.5) | 42.9 (37.4–48.4) |
| Specificity (95% CI) | 98.4 (97.7–99.0) | 97.7 (96.8–98.4) | 97.9 (97.0–98.6) | 98.6 (97.9–99.2) | 98.1 (97.3–98.7) | 96.7 (95.6–97.5) | 98.4 (97.6–99.0) |
| PPV (95% CI)          | 91.9 (88.1–94.8) | 82.7 (76.6–87.7) | 78.3 (70.7–84.8) | 88.2 (82.3–92.6) | 85.3 (79.5–90.0) | 57.0 (47.4–66.3) | 85.5 (79.1–90.5) |
| NPV (95% CI)          | 95.6 (94.4–96.6) | 89.7 (88.1–91.2) | 87.1 (85.4–88.6) | 89.1 (87.5–90.6) | 89.7 (88.1–91.1) | 84.3 (82.5–86.0) | 88.5 (86.9–90.0) |

TP 262 162 112 149 163 65 141
TN 1454 1439 1447 1453 1441 1415 1450
FP 23 34 31 20 28 49 24
FN 67 165 215 177 166 263 188

Study Part II

RDTs kept at 35°C and 45°C for 15, 30, 60, 90 and 100 days and at 60°C for 48 hours for heat stability test in the field. Seventy five clinically suspected malaria cases were tested on 15, 30, 60 and 90 days, and 50 clinically suspected cases were tested on 100 days and at 60°C for 48 hours intervals.

Results of heat stability testing was shown in Table 3 & 4. Experiments showed that sensitivity of most of the RDTs for *P. falciparum* was very good (>90) both at 35°C and 45°C up to day 90 when compared with RDTs kept at room temperature. However, a sharp decline in sensitivity was recorded on day 100 at 35°C and 45°C by most of the RDTs. On the contrary all RDTs kept at 60°C for 48 hours showed no decline in the diagnostic performance.

For *P. vivax*, the sensitivity of FIRST RESPONSE® was very good up to 90 days (100%) and a decline was noticed on day 100 (92%). Parascreen also performed well up to day 90 at 35°C (88%). However, a sharp decline in sensitivity was observed on day 90 at 45°C (75%). While other RDTs showed a steady decline in sensitivity from day 60 onwards (Table 4). The overall agreement and Kappa values between pairs of observers were very good for both at 35°C and 45°C for *P. falciparum*. However, Kappa values was not good for some RDTs for *P. vivax* especially on days 90 and 100 (Figure 3).
Comparative Evaluation of Bivalent Malaria RDTs

**P. falciparum**

| Test | Sensitivity (%) |
|------|-----------------|
| FalciVax | ![Graph](image1) |
| FIRSTRESPONSE | ![Graph](image2) |
| Genomix | ![Graph](image3) |
| NecViParum | ![Graph](image4) |
| ParaHIT Total | ![Graph](image5) |
| parascreen | ![Graph](image6) |

**P. vivax**

| Test | Sensitivity (%) |
|------|-----------------|
| FalciVax | ![Graph](image7) |
| FIRSTRESPONSE | ![Graph](image8) |
| Genomix | ![Graph](image9) |
| NecViParum | ![Graph](image10) |
| ParaHIT Total | ![Graph](image11) |
| parascreen | ![Graph](image12) |

Parasite Density (per cmm)

- Sensitivity (%)
- 95% CI
Discussion

A number of studies on RDTs have been conducted, although measures of accuracy have varied widely, as a result of differences in methodology, study site epidemiology and type of RDT used i.e. measures of accuracy have varied widely, as a result of differences in methodology, study site epidemiology and type of RDT used i.e.

Figure 2. Parasite density and Sensitivity of seven RDTs of P. falciparum and P. vivax.

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The present study assessed the performance of various bivalent RDTs in field population. Overall, highest sensitivity for P. falciparum was >95% and for P. vivax ≥80%. The high frequency of positive smears in this study is consistent with previous studies in Central India [30]. Likewise the sensitivity and specificity estimates are consistent with previous studies [10]. Further, when the results of this study was compared with WHO, FIND and CDC product testing of RDTs, where the evaluation was performed against a standardized panel of cultured P. falciparum and frozen blood samples (200–2000 parasites/μl of blood) by experienced techni-

Table 3. Sensitivity of 7 different RDTs at 35°C, 45°C and 60°C for P. falciparum against Room Temperature.

| Time Intervals | Temp | FIRST RESPONSE<sup>®</sup> | Parafilm<sup>®</sup> | ParaHIT<sup>®</sup> Total | Falcivax | SD BIOLINE | GENOMIX | NecVIPARUM |
|---------------|------|-----------------|----------------|----------------|--------|------------|---------|------------|
|               |      | Sens. (95%CI) | Spec. (95%CI) | Sens. (95%CI) | Spec. (95%CI) | Sens. (95%CI) | Spec. (95%CI) | Sens. (95%CI) | Spec. (95%CI) |
| 15 days       | 35°C | 93 (92-100)   | 100 (95-100)  | 89 (85-94)    | 90 (86-95)    | 72 (66-80)  | 91 (87-96)  | 84 (78-91)  | 87 (81-94)  |
|               | 45°C | 93 (92-100)   | 97 (93-100)   | 85 (80-91)    | 93 (88-98)    | 75 (70-80)  | 93 (88-98)  | 82 (76-89)  | 87 (81-94)  |
|               | 60 days | 95 (92-100) | 100 (95-100) | 98 (94-104)  | 94 (90-100)  | 98 (94-100) | 95 (91-100) | 92 (88-98)  | 100 (96-100) |
| 30 days       | 35°C | 95 (92-100)   | 100 (95-100)  | 98 (94-104)  | 94 (90-100)  | 95 (91-100) | 92 (88-98)  | 95 (91-100) | 89 (85-96)  |
|               | 45°C | 97 (94-100)  | 100 (95-100)  | 93 (89-97)    | 98 (93-100)   | 93 (88-98)  | 100 (96-100) | 87 (82-94)  | 95 (90-98)  |
|               | 60 days | 97 (94-100) | 100 (95-100) | 92 (88-96)   | 93 (89-98)    | 82 (78-94)  | 100 (96-100) | 77 (72-93)  | 97 (92-99)  |
| 90 days       | 35°C | 96 (93-100)   | 100 (95-100)  | 96 (92-100)  | 94 (90-100)  | 95 (91-100) | 92 (88-98)  | 95 (91-100) | 90 (86-98)  |
|               | 45°C | 94 (91-100)   | 100 (95-100)  | 94 (90-100)  | 96 (92-100)  | 92 (88-98)  | 100 (96-100) | 87 (82-94)  | 95 (90-98)  |
|               | 60 days | 96 (93-100) | 100 (95-100) | 94 (90-100)  | 92 (88-98)    | 93 (89-98)  | 100 (96-100) | 89 (85-96)  | 90 (86-98)  |
| 100 days      | 35°C | 88 (85-95)    | 100 (95-100)  | 88 (84-93)    | 92 (88-97)    | 88 (84-93)  | 100 (96-100) | 87 (82-94)  | 95 (90-98)  |
|               | 45°C | 76 (73-80)    | 100 (95-100)  | 84 (80-88)    | 96 (92-100)   | 82 (78-94)  | 100 (96-100) | 84 (80-96)  | 82 (78-94)  |
|               | 60 days | 55 (52-58)  | 100 (95-100)  | 64 (60-68)    | 96 (92-100)   | 74 (70-80)  | 100 (96-100) | 64 (60-70)  | 82 (74-86)  |
| 48 hr         | 60°C | 92 (89-95)    | 100 (95-100)  | 92 (88-96)    | 95 (91-99)    | 92 (88-96)  | 100 (96-100) | 90 (86-100) | 92 (88-100) |

Heat stability is vital to maintaining sensitivity of the test in the field [22], [30]. HRP-2 is a very stable antigen [31], while pLDH may degrade during long storage [11], [22]. Wide variations in stability between various RDTs were recorded in this study. The lacks of quality control of RDTs present a risk to patients through incorrect diagnosis and inappropriate anti-malarial treatment [32].
As a result for procurement, it is essential that careful consideration be given to stability results to ensure that RDTs work under extreme temperature. All the RDTs in this evaluation were packaged in individual envelopes that contain a desiccant. This allows the health worker to open the envelope of a test at the time of use in field limiting exposure to high humidity [12] as the field trial for stability testing was carried out during peak rainy season. The stability testing results presented here provide assessment of both, stability of the RDT and also the quality of its packaging [12]. However, there are some potential limitations in generalizing our results to predict the success of implementing RDTs at high temperature. Though temperature was held constant in this evaluation, humidity was not maintained. Temperature and humidity in field fluctuate with time of day and season and 100% humidity in field fluctuate with time of day and season and 100% storage may not accurately predict long term stability under field conditions. Loss of parasite detections over this period indicates that chances of decline in sensitivity cannot be overlooked [12]. It is worthwhile to mention here that field trial was carried out during July–March thus all 3 seasons i.e. monsoon, autumn and summer were covered. An additional limitation of this study was that highly trained individual performed all the testing in this evaluation. In field settings malaria RDTs will often be used by health workers with limited training and supervision. Temperature up to 45°C is likely in uncontrolled storage in tropical countries and temperature may further increase during transport [33]. The overall agreement and Kappa values between pairs of observers were excellent for both positive and negative results. Likewise, for heat stability testing for P. falciparum generally agreement and Kappa values were good for all RDTs however, for P. vivax the Kappa was not good for most RDTs on day 90 and 100. Thus the use of RDTs widely in the programme will require considerable regulation and quality control [34]. Further research is required to determine why some RDT’s examined were more susceptible to heat stress in order to improve their temperature stability.

In view of this, while malaria RDTs can be applied in a number of settings, the greatest potential for impact on public health is in extension of access to accurate, parasite based diagnosis to malaria regions and populations where good quality microscopy based testing is impractical to maintain, making possible the implementation of recent WHO/NVBDCP recommendation on parasite based diagnosis prior to anti-malarial therapy [25], [35]. However, if the RDTs are to replace microscopy in field for malaria diagnosis, they must be able to work with high level of reliability at high ambient temperature. Diagnostic testing by microscopy or RDT to a level of 200 parasites/μl will reliably detect nearly all clinically relevant infections in malaria endemic areas [10], [36]. However, as some countries move towards elimination, population immunity will be decreased and it will increasingly important to use diagnostic tests that detects low parasite densities [17], [37], [38]. Therefore, the ability to detect low parasite density infections

### Table 4. Sensitivity of 7 different RDTs at 35°C, 45°C and 60°C for P. vivax against Room Temperature.

| Time Intervals | Temp | FIRST RESPONSE® | Parascreen® | ParaHIT® Total | FalciVax | SD BIOLINE | GENOMIX | NecVIPARUM |
|---------------|------|-----------------|-------------|----------------|-----------|------------|---------|------------|
|               |      | Sens. (95%CI)   | Sens. (95%CI) | Sens. (95%CI)  | Sens. (95%CI) | Sens. (95%CI) | Sens. (95%CI) | Sens. (95%CI) |
| 15 days       | 35°C | 100 (86-100)    | 100 (93-100) | 93 (61-95)     | 71 (42-92)  | 91 (48-89)  | 90 (45-92) | 93 (48-90)  |
|               | 45°C | 96 (80-100)     | 97 (89-100)  | 94 (66-97)     | 91 (42-92)  | 90 (48-89)  | 90 (45-92) | 97 (45-92)  |
| 30 days       | 35°C | 97 (86-100)     | 100 (91-100) | 98 (60-98)     | 97 (44-90)  | 100 (88-100)| 99 (45-92) | 97 (84-94)  |
|               | 45°C | 94 (81-99)      | 97 (86-100)  | 97 (60-98)     | 97 (33-82)  | 97 (81-97)  | 96 (45-92) | 94 (83-95)  |
| 60 days       | 35°C | 100 (86-100)    | 99 (89-100)  | 99 (56-100)    | 97 (48-98)  | 99 (89-100)| 99 (52-100) | 99 (52-100) |
|               | 45°C | 100 (86-100)    | 99 (89-100)  | 99 (56-100)    | 99 (48-98)  | 100 (89-100)| 99 (52-100) | 99 (52-100) |
| 90 days       | 35°C | 100 (87-100)    | 99 (93-100)  | 99 (47-100)    | 99 (14-79)  | 98 (85-98)  | 99 (52-100) | 99 (52-100) |
|               | 45°C | 100 (87-100)    | 99 (93-100)  | 99 (35-97)     | 99 (17-79)  | 98 (85-98)  | 99 (52-100) | 99 (52-100) |
| 100 days      | 35°C | 99 (82-99)      | 95 (90-100)  | 99 (15-95)     | 99 (1-91)   | 99 (86-100)| 99 (18-90)  | 99 (86-100) |
|               | 45°C | 99 (62-100)     | 97 (82-99)   | 99 (5-80)      | 99 (1-91)   | 99 (83-99)| 99 (18-90)  | 99 (88-100) |
| 48 hr         | 60°C | 100 (63-100)    | 100 (92-100) | 100 (29-100)   | 99 (80-100) | 100 (86-100)| 99 (80-100) | 100 (85-100) |

As a result for procurement, it is essential that careful consideration be given to stability results to ensure that RDTs work under extreme temperature. All the RDTs in this evaluation were packaged in individual envelopes that contain a desiccant. This allows the health worker to open the envelope of a test at the time of use in field limiting exposure to high humidity [12] as the field trial for stability testing was carried out during peak rainy season. The stability testing results presented here provide assessment of both, stability of the RDT and also the quality of its packaging [12]. However, there are some potential limitations in generalizing our results to predict the success of implementing RDTs at high temperature. Though temperature was held constant in this evaluation, humidity was not maintained. Temperature and humidity in field fluctuate with time of day and season and 100 days storage may not accurately predict long term stability under field conditions. Loss of parasite detections over this period indicates that chances of decline in sensitivity cannot be overruled [12]. It is worthwhile to mention here that field trial was carried out during July–March thus all 3 seasons i.e. monsoon, autumn and summer were covered. An additional limitation of this study was that highly trained individual performed all the testing in this evaluation. In field settings malaria RDTs will often be used by health workers with limited training and supervision. Temperature up to 45°C is likely in uncontrolled storage in tropical countries and temperature may further increase during transport [33]. The overall agreement and Kappa values between pairs of observers were excellent for both positive and negative results. Likewise, for heat stability testing for P. falciparum generally agreement and Kappa values were good for all RDTs however, for P. vivax the Kappa was not good for most RDTs on day 90 and 100. Thus the use of RDTs widely in the programme will require considerable regulation and quality control [34]. Further research is required to determine why some RDT’s examined were more susceptible to heat stress in order to improve their temperature stability.

In view of this, while malaria RDTs can be applied in a number of settings, the greatest potential for impact on public health is in extension of access to accurate, parasite based diagnosis to malaria regions and populations where good quality microscopy based testing is impractical to maintain, making possible the implementation of recent WHO/NVBDCP recommendation on parasite based diagnosis prior to anti-malarial therapy [25], [35]. However, if the RDTs are to replace microscopy in field for malaria diagnosis, they must be able to work with high level of reliability at high ambient temperature. Diagnostic testing by microscopy or RDT to a level of 200 parasites/μl will reliably detect nearly all clinically relevant infections in malaria endemic areas [10], [36]. However, as some countries move towards elimination, population immunity will be decreased and it will increasingly important to use diagnostic tests that detects low parasite densities [17], [37], [38]. Therefore, the ability to detect low parasite density infections
reliably therefore, remains important as malaria elimination initiative is increasing in several countries.

Nevertheless, the challenges associated with establishing the routine use of RDTs in rural remote setting where microscopy could not be established should be tackled carefully as the distribution of RDTs and antimalarials must occur hand in hand to ensure effective case management of febrile disease. These results also suggest that the quality of RDTs should be regulated and monitored more closely. We also noticed a wide range in pricing for RDTs ranging from 0.70$ to 2$. However, high price of RDTs is not an assurance of good performance. Therefore, continued search and eventually introducing other alternative and highly sensitive low cost malaria diagnostic methods should also be explored which are capable of detecting low parasitaemia at high ambient temperature.

Supporting Information

Table S1 Details of bivalent Rapid Diagnostic Tests. (DOC)

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Author Contributions

Conceived and designed the experiments: NS RS. Performed the experiments: PKB MPS SM MMS. Analyzed the data: NS PKB MPS SM MMS RKS RS. Wrote the paper: NS PKB MPS RKS RS.

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