Evaluation of two malaria rapid diagnostic tests quality assurance (mRDT's QA) methods in peripheral health facilities, rural Tanzania.

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Evaluation of two malaria rapid diagnostic tests quality assurance (mRDTs QA) methods in peripheral health facilities, rural Tanzania

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EXECUTIVE SUMMARY:

Background: WHO recommends confirming suspected malaria cases before initiation of treatment. Due to the limited availability of quality microscopy services, this recommendation has been followed with increased use of antigen-detecting malaria rapid diagnostic tests (mRDTs) in many malaria endemic countries. With the increased use of mRDTs, the need for a thorough mRDT quality assurance (RDT QA) method has become more apparent. One of the WHO recommendations for RDT QA is to monitor the tests in field use monthly, by comparing mRDT results to reference microscopy. This study was carried out to monitor mRDT performance in selected health facilities using two quality assurance methods; first based on reference microscopy and second based on detection of parasite DNA by real time quantitative PCR (qPCR) on dried blood spots (DBS); as well as assessing the cost and timeliness of the two QA methods.

Methods: Blood samples were collected from patients undergoing a rapid test for malaria for two to three consecutive days per month, for five months, in 12 health facilities in Iringa rural and Mufindi districts. The health workers were instructed to label RDT cassettes, blood smear slides, and filter papers for DBS with matching unique ID stickers. A sticker was also placed in the log book where RDT results were recorded. Blood smears (BS) were first read at the district hospital (BS1) and then transported to Bagamoyo for a reference reading at the IHI- Bagamoyo laboratory (BS2). A third BS reader (BS3) was consulted from Muhimbili University of Health and Allied Sciences (MUHAS) in case of discordant results between BS1 and BS2. Molecular analysis involved extraction of parasite DNA from DBS samples using a QIAamp DNA Mini Kit. Sample DNA aliquots were compared against standard solutions with parasite DNA diluted 10-fold to give a parasitemia ranging from 200,000/µL to 20/µL. About 20% of the study DNA aliquots were sent to the CDC laboratory in Atlanta in order to validate qPCR results performed at the Bagamoyo laboratory.

Data were entered in Microsoft Access (Microsoft Corporation, 2006) and analyzed in STATA 10 (StataCorp, Texas USA). Because of the known limitations of mRDTs to detect parasitemia below 200 parasites/µL, BS and PCR results greater than or equal to 5 parasites/200 WBC or 200 parasites/µL were considered positive in comparisons with mRDT performance. In the univariate analysis, proportions of positive tests were compared among the three types of tests: mRDT, microscopy and qPCR. Microscopy readings were categorized into 3 groups; BS1, BS2 and /or BS summary which is an average of BS1 and BS2. In case of discordant results between BS1 and BS2, a third reader- BS3 was consulted. Chi-squared test was done to assess differences in proportion of positive tests per district; whereas McNemar's test was

used to assess the difference in test positivity by type of test. Kappa statistic was used to quantify the strength of the agreement between tests results.

In addition, we examined health workers performance of the testing procedure when attending patients at a health facility, using a predefined checklist. Towards the end of the study, an evaluation of health worker acceptability was carried out to assess preferences between the two RDT QA methods.

Results: We received 2369 samples and 2324 (98%) had complete information. mRDTs had the highest positivity rate (6.5%). The proportion of positive tests by all types of tests was slightly higher in Iringa DC, but only qPCR and BS2 showed statistically significant differences in positivity rate between the two districts, where Iringa DC had more positive tests than Mufindi DC ($p<0.05$). When qPCR was a gold standard, mRDTs had higher sensitivity (68.6%, 95%CI: 55.0-79.7) than microscopy (53.7%, 95%CI: 38.7-68.0) but highest mRDT sensitivity was achieved with comparison to microscopy (85.3%, 95%CI: 70.0-93.6). All tests had higher inter-observer agreement than would be expected by chance. Substantial high inter-observer agreement (kappa =0.75; $p<0.001$) was seen amongst the microscopists i.e. district's quality assurance officers and the reference microscopy readings.

Assessment of the time needed to process BS at the district level revealed that, smears at district level took on average 8 days (min 2 to max 33) to be processed and provide feedback; but up to an average of 44 days (min 19 to max 98) to get a second reading. Many health workers were aware that the use of mRDTs was due to changes in treatment policy (11/30), and patients who qualify for the test are those suspected to have malaria. Majority (16/30) related assessment of control line as a measure of test accuracy and suggested the use of microscopy for quality control of mRDT results (15/30). Their major concerns were mRDTs' inability to give parasite count, stock-out of the tests kits in their working areas and the frequency of negative results.

Challenges: This evaluation encountered several challenges, among them were 1. Poor quality of blood smears made at health facilities, especially dispensaries, which do not have laboratory services. 2. About 3.5% of BS1 slides could not be processed for BS2 because they were damaged during transportation and/or poor quality of smears. This accounts for the small difference in the numbers of BS assessed between two readers. 3. We were not able to prepare standard concentration solutions for qPCR analysis in the country. 4. Problems with PCR machine and inability to repair it that necessitated shipment of the machine, to and from, the manufacturers in Europe (Germany).

Due to these challenges, qPCR results were not available until after specimen collection had ended. .

Conclusions: In this study malaria positivity was higher with mRDTs than microscopy and qPCR for the 200 parasites/µL lower boundary of positivity threshold. This could either be due to the strict lower cut-off point for microscopy and qPCR parasite density or higher false positivity of mRDTs due to persistent antigen in blood, errors in mRDTs performance or other patient's characteristics. When qPCR was taken as gold standard, mRDTs showed better sensitivity than microscopy, but when microscopy was regarded as a gold standard, mRDTs showed higher sensitivity than with qPCR. However, results of qPCR demonstrated a better correlation (inter-observer agreement) with those of microscopy than with mRDTs.

The challenges of performing qPCR, as observed in this evaluation, make it unsuitable for quality assurance of mRDTs in routine care, Tanzania. The high inter-observer agreement between districts' and reference microscopists (K=0.75) and higher tests performances of BS1 when BS2 was a comparator, demonstrates the competence shown by district's technicians/ technologists to suffice their involvement as reference microscopists for quality assurance of mRDTs in their respective districts. This is also complimented by a fact that, both BS1 and BS2 had more similar performance when qPCR was taken as a gold standard.

In this setting, a microscopy-based quality assurance system to assess mRDT performance in routine use may be a practical and suitable method. However, long distance transportation of smears should be avoided.

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INTRODUCTION:

The 2010 World Malaria Report declares that there have been tremendous achievements in the reduction of malaria burden worldwide, particularly in endemic countries [WHO, 2010]. This has mainly been a result of increased coverage of malaria control interventions such as the use of insecticide-treated mosquito nets (ITNs), indoor residual spraying (IRS), larviciding and use of effective treatments based on artemisinin combination therapy (ACTs). The WHO recommendation of confirming suspected malaria cases before treatment is being implemented in many malaria endemic countries in sub-Saharan Africa and elsewhere [Harvey SA et al 2008, Zurovac D et al 2008]. The use of antigen-detecting rapid diagnostic tests (RDTs) forms a vital part of this strategy [WHO- TDR 2011].

While parasite-based malaria diagnosis is increasing, the effect of misclassification of clinical disease due to incorrect test results is also becoming more apparent. There is need for a thorough RDT quality assurance (RDT QA) method with a shorter turn-around time for assessment of malaria confirmatory tests in routine use. One of the WHO recommendations for RDT QA is to monitor the tests in field use monthly, by comparing mRDT results to reference microscopy [WHO, 2008]. In this recommendation, each health facility using mRDTs is expected to submit blood smears from 20 mRDT positive and 20 mRDT negative patients which may not be feasible in areas of low malaria transmission.

In view of this recommendation, the Ifakara Health Institute (IHI) in collaboration with the US Centers for Disease Control and Prevention (CDC) undertook a study to assess two methods of quality assurance (QA) for malaria rapid diagnostic tests (mRDT) used in routine care of suspected malaria patients in early 2010. The study was initiated to assist the National Malaria Control Programme (NMCP) efforts of expanding malaria diagnostic capacity by assessing a suitable mRDT quality control method that can be adopted nationwide. The Tanzanian Ministry of Health and Social Welfare through NMCP, is introducing mRDTs to all levels of care, in order to reduce inappropriate use of the first-line antimalarial drugs. In their phased mRDT roll out, the NMCP introduced the tests in Iringa, Kagera and Coast regions in 2009. All regions are expected to have received mRDT training and roll out by mid 2012.

The aim of this study therefore, was to monitor mRDT performance in selected health facilities in two districts (Iringa and Mufindi) using two QA methods; first based on reference microscopy and second based on detection of parasite DNA by real time quantitative PCR (qPCR) on dried blood spots (DBS). We also aimed at assessing the cost and timeliness of the two QA methods.

METHODS:

Location: 12 health facilities from Iringa region (6 in Mufindi- *Mafinga District Hospital, Malangali Health Center, Igomaa Dispensary, Sadani Dispensary, Kibao Health Center, Usokami Health Center* and 6 in Iringa rural district- *Tosamaganga Designated District Hospital, Mlowa Dispensary, Idodi Health Center, Kimande Health Center, Ifunda Dispensary and Ilambilole Dispensary*) were selected in collaboration with the District Medical Officer (DMO) and the National Malaria Control Program (NMCP) to participate in this evaluation.

Sample collection*:* Samples were collected from patients undergoing a malaria rapid test for two to three days per month, for the 5 months of data collection. To perform a rapid test, health worker must use a lancet to collect a peripheral blood sample. The blood is then collected in a transfer device (either a capillary tube or loop) to be placed in the appropriate well on the RDT where it is absorbed by the nitrocellulose paper. An amount less than 1 drop of blood (typically 5-10µL) is sufficient. From the same finger prick we collected 2-3 drops of blood for a blood smear and 2-4 drops of blood for a dried blood spot (DBS) – patients did not undergo a second blood draw.

Training: Health workers at all participating facilities were trained to perform RDTs by the local district council health management team (CHMT), during the national roll out. The types of mRDT used were those available from the facilities through the existing procurement system (Paracheck Pf ® - from Orchid Biomedical system and ParaHit® from Span Diagnostics). For the study purposes, we trained health workers on how to perform a thick blood smear and collect a DBS. Log books and unique ID stickers were provided to record RDT results and for specimen tracking. Each facility received training on how to appropriately collect, label and store specimens.

Data collection: Study facilities received monthly visits from the district QA supervisor, who is also a laboratory technician. Once a month, on the 2 days prior to the arrival of the supervisor, thick blood smears and DBS were collected from all patients receiving RDTs at the health facility. The health workers were instructed to label RDT cassettes, blood smear slides, and DBS papers with matching unique ID stickers. A sticker was also placed in the log book where RDT results were recorded.

At the facility, the QA supervisor collected 40 BS/DBS pairs made the previous two days. BS samples were sent direct to the district laboratory technician to be stained and read as BS1. The technicians who read BS1 at the district had recently undergone microscopy refresher training at the National Institute for Medical

Research (NIMR) headquarters, Dar es Salaam. After reading, all blood smears along with their matching DBS were sent to Dar es Salaam to the study investigator, who, in turn send them to IHI – Bagamoyo laboratory to be processed for second reading (BS2). Any discordant readings between BS1 and BS2 warranted a third reading, BS3, by a senior technician from the Muhimbili University of Health and Allied Sciences (MUHAS). DBS samples were processed and analyzed in Bagamoyo laboratory as qPCR1. A proportion of DNA extracts were sent to the CDC laboratory in Atlanta for validation of Bagamoyo results, referred to as qPCR2.

Health workers assessment: Three times in the study the Tanzanian investigator and district QA supervisor assessed availability of mRDT devices in respective health facilities, and recorded health workers' performance of a testing procedure using a standardized checklist. At the end of the study, we provided feedback to health workers and conducted a qualitative enquiry to assess health workers preferences and acceptability of the two QA methods under study.

Sample processing: At the district laboratory, BS were stained using 10% Giemsa solution buffered to pH 7.2 for 8 -10 minutes in Iringa DC or 15 minutes in Mufindi. When a dilution of 5% was used, staining took 30 minutes. A BS was declared negative after reading 100 high power fields. For positive smears, parasites were counted in reference to 200 white blood cells (WBCs).

Molecular analysis involved extraction of parasite DNA from DBS samples using a QIAamp DNA Mini Kit (QIAGEN, Valencia, CA- (Qiagen method)). The DNA was aliquoted and stored at -20°C until used. *P. falciparum* (3D7) was cultured in the CDC laboratory, Atlanta, GA. The cultures were synchronized by the sorbitol method to select for the ring stage parasites with single nuclei and therefore can be reproducibly used for quantification of DNA. A thin smear was made, stained with Giemsa and the percentage parasitemia determined. The number of parasites/µL was determined by counting the total number of RBCs/µL using a coulter counter and percentage parasitemia data. This was regarded as the standard DNA solution and was diluted 10-fold to give a parasitemia range from 200,000/µL to 20/µL. Sample DNA aliquots (from RDT QA study) with unknown parasite concentrations were compared or run against the known standard DNA solutions in order to detect the presence of parasite DNA. About 20% of the study sample DNA aliquots were sent to CDC to validate qPCR results at Bagamoyo laboratory.

qPCR assay was performed using commercially available PCR buffer and taq polymerase (New England Biolabs, Ipswich, MA). Primers and a HEX-labeled Taqman probe to the *P. falciparum* beta-tubulin gene

were designed and used as previously described [6]. DNA amplification was performed using the following cycling conditions: 95°C for 5 minutes, followed by 50 cycles of 95°C for 20 seconds and 58°C for 1 minute using the Mx3005p real time PCR machine with MxPro QPCR software (Stratagene). The standard DNA was included in each experiment together with the field samples (unknowns). Each sample and standard was run in triplicate, and all experiments were repeated to ensure consistency. The standard 3D7 DNA was used to prepare a standard curve which was used to quantify the amount of parasites in the field samples.

***Data processing*:** Data were entered in Microsoft Access and analyzed in STATA 10. Qualitative responses were coded and summarized in Microsoft Excel. BS1 and BS2 readings, RDT and PCR results were compared by the study investigators. For comparison, BS and PCR results considered positive were those at or above 5/200 WBC or 200/ µL as this was considered the potential limit of detection of most rapid diagnostic tests for malaria [7]. Statistical analysis involved description of tests and test results by health facility type and by district. In the univariate analysis, proportions of positive tests were compared against the three types of tests; RDT, microscopy and qPCR. Microscopy readings (BS1 and BS2 or BS1 and BS2/ BS3) were summarized to get one variable for microscopy results (BS summary, here referred to as BSsum). Chi-squared test assessed differences in proportion of positive tests by district. Cross tabulation between test results was used to measure tests' sensitivity, specificity, positive and negative predictive values first for RDT, taking BS and qPCR as gold standard, and then assessing BS with qPCR as gold standard. McNemar's test was used to assess the difference in proportion of positive results by type of test. Kappa statistic was used to quantify the strength of the tests' agreement.

Ethical clearance: This work was granted ethical permit by the IHI- Ethical review board. The protocol was amended in August, 2010 to allow movement of extracted *Plasmodium falciparum* DNA aliquots to CDC Laboratory, Atlanta, USA. The sample transportation (import/ export) permit certificate dated September 1st, 2010, was issued by the Tanzania Private Health Laboratories Board for this purpose.

RESULTS:

Samples were collected in January, February, March, May and August 2010 from the 12 participating health facilities. A total of 2400 samples were expected from all health facilities. We received 2369 samples and 2324 (98%) had complete patients and test results information. During data entry, a number of samples were found to have duplicated sample IDs due to a printing error of sticker labels. 487 (20.9%) records were removed from the analysis. As well, 3.5% (60) slides with BS1 results could not be reviewed with the second reader (BS2), due to transportation challenges and poor quality of smears. Total samples included in the analysis by district and by health facility is presented in table 1.

About 1000 DNA aliquots were sent to CDC laboratory in Atlanta. Results of 412 samples were available; which excluded samples that had too little DNA extracts and/ or indeterminate results. Only 300 results could be compared with other tests, after removing duplicate IDs.

Table 1: Total tests collected and analyzed by District and Health facility

District	Name of Health facility	Expected samples	Total RDT	Total BS1	Total BS2	Total DBS
Iringa DC	Idodi Health Center	200	164	164	164	161
Iringa DC	Ifunda Dispensary	200	196	195	196	156
Iringa DC	Ilambilole Dispensary	200	184	180	170	156
Iringa DC	Kimande Health Center	200	194	158	194	166
Iringa DC	Tosamaganga Hospital	200	158	88	109	115
Iringa DC	Mlowa Dispensary	200	160	156	156	158
Mufindi DC	Igomaa Dispensary*	200	92	92	91	91
Mufindi DC	Kibao VA Health Center**	200	154	154	154	76
Mufindi DC	Mafinga Hospital	200	161	161	142	118
Mufindi DC	Malangali Health Center**	200	152	140	55	101
Mufindi DC	Sadani Health Center*	200	60	60	60	59
Mufindi DC	Usokami Health Center	200	162	162	159	149
Total/ (% out of expected)		**2400 (100)**	**1837 (76.5)**	**1710 (71.3)**	**1650 (68.7)**	**1506 (62.7)**

*= Some samples had identical unique Id's, hence removed from the analysis

**= Some samples were not sent for reference reading and/ or further analysis in Dar es Salaam

***Malaria positivity*:** Malaria positivity differed between the two districts as well as by test type. MRDTs had the highest positivity rate but the difference was not statistically significant between the districts (Table 2a). Proportion of positive tests by type of RDT device as well as parasite count also did not differ between the districts (Tables 2b and 2c). Based on microscopic analysis of blood smears, Iringa DC had more positive tests as compared to Mufindi DC. The difference was statistically significant with the reference microscope reading (BS2). This was also shown by qPCR results, where the positivity rate for Iringa DC was closer to that of mRDTs, than microscopy- Table 2.

Table 2a: Number of positive tests between Iringa DC and Mufindi DC [p*: Chi-squared]

Type of tests (n/N)	Iringa DC	Mufindi DC	p*-value
RDT (N=120/1839)	62/1096 (5.7%)	58/747 (7.7%)	p=0.07
BS1 (N=50/1712)	34/977 (3.5%)	16/735 (2.2%)	p=0.11
BS2 (N=42/1652)	33/1025 (3.2%)	9/627 (1.4%)	p=0.025
qPCR (N=51/1508)	47/914 (5.2%)	4/594 (0.7%)	p<0.001
qPCR2 (N=15/300)	15/193 (7.7%)	0/107	p=0.003

Table 2b: Number of mRDT tests performed and the proportion of positive tests by mRDT type [*p-value (Chi-squared test) = 0.001]

Type of RDT (total tests)	Total mRDT in Iringa DC (%)	Total mRDTs in Mufindi DC (%)	Proportion of positive tests (%)
Paracheck (n=785)	80 (10)	705 (90)	68 (8.7)
ParaHit (n=1054)	1012 (96)	42 (4)	52 (4.9)
Total (N=1839)	1092 (59.4)	747 (40.6)	120 (6.5)

Table 2c: Levels of Parasitemia by type of test

Category (per 200 WBC)	BS1 (%)	BS2 (%)	qPCR1 (%)
0	1662 (97.1)	1609 (97.4)	1455 (96.5)
5-500	41 (2.4)	30 (1.8)	25 (1.7)
>500	9 (0.5)	13 (0.8)	28 (1.8)
Total	1712 (100)	1652 (100)	1508 (100)

***Tests performance*:** The ability of the tests to detect a positive blood sample was higher with mRDTs (85.3%, 95%CI: 70- 93.6) when microscopy was taken as a gold standard (Table 3). Comparing to qPCR, mRDTs had a sensitivity of 68.6% (95%CI: 55-79.7) and the lowest when microscopy was compared with qPCR as a gold standard (53.7%, 95%CI: 38.7- 68). On the other hand, district microscopy had good

sensitivity when compared to reference microscopy readings 84.6% (95% CI: 70.3- 92.8). Likewise, positive predictive value was better with all microscopic analysis of blood smear than with rapid tests. Specificity and negative predictive values were considerably higher with both tests- Table 3.

Table 3: Tests Performance : Sensitivity, Specificity and Predictive values

[*= Gold standard test]

Test combination (n)	Sensitivity (95%CI)	Specificity (95%CI)	PPV (95%CI)	NPV (95%CI)
RDT/ BS1* (1710)	82% (69.2 - 90.2)	96% (96.0 – 97.0)	38% (29.7- 47.8)	99.4% (98.9 - 99.7)
RDT/ BS2* (1650)	78.6% (64.1 - 88.3)	95.6% (94.5 - 96.5)	32.0% (23.8 - 41.6)	99.4% (98.9 - 99.7)
RDT/ Bs-sum* (1519)	85.3% (70 - 93.6)	95.9% (94.7 - 96.7)	31.5% (23.0 - 41.6)	99.7% (99.2 - 99.9)
RDT/ qPCR* (1506)	68.6% (55 - 79.7)	95.8% (94.7 - 96.7)	36.5% (27.5 - 46.4)	98.9% (98.2 - 99.3)
BS1/ BS2* (1506)	84.6% (70.3 - 92.8)	99% (98.4 - 99.4)	68.8% (54.7 - 80.1)	99.6% (99.1 - 99.8)
BS1/ qPCR* (1380)	57.8% (43.3 - 71)	98.9% (98.2 - 99.3)	63.4% (48.1 - 76.4)	98.6% (97.9 - 99.1)
BS2/ qPCR* (1310)	54% (40.4 - 67)	99.2% (98.6 - 99.6)	73% (57.0 - 84.6)	98.3% (97.4 - 98.8)
Bs-sum/ qPCR* (1266)	53.7% (38.7 - 68)	99.4% (98.9 - 99.7)	75.9% (58.0 - 78.8)	98.5% (97.7 – 99.0)
qPCR1/ qPCR2* (270)	88.4% (84.0- 91.8)	82% (52.0- 95.0)	99.1% (97.0- 99.8)	23.1% (12.6- 38.3)

***Inter-observer agreement between tests*:** All tests had higher inter-observer agreement than what would be expected by chance. High inter-observer agreement was seen amongst the microscopist i.e. district's quality assurance officers and the reference microscopy readings (K=0.75), followed by a comparison between any microscopy reading (BS1/2/sum and qPCR), Table 4. RDT had only moderate agreement with both microscopy and qPCR. Despite a very good Pearson's correlation between the two qPCR readings (0.9), the kappa coefficient recorded only a fair agreement between them.

Table 4: Proportion of agreements between two tests: McNemar's comparison

Tests combination	Observed agreement	Expected agreement	p*-value	Kappa statistic**	Correlation
RDT - BS1 (n=1712)	95.6%	91.2%	p<0.001	0.50	0.54
RDT - BS2 (n=1652)	95.2%	91.5%	p<0.001	0.43	0.48
RDT- Bssum (n=1555)	95.6%	92.2%	p<0.001	0.44	0.50
RDT – qPCR (n=1508)	94.9%	90.7%	p<0.001	0.45	0.47
BS1 - BS2 (n=1508)	98.6%	94.5%	p=0.05	0.75	†0.76
BS1 – qPCR (n=1427)	97.6%	94.2%	p=0.6	0.59	0.59
BS2 – qPCR (n=1362)	97.6%	93.8%	p=0.035	0.61	0.61
Bssum – qPCR (n=1309)	98.0%	94.7%	p=0.029	0.62	0.62
qPCR - qPCR2 (n=270)	88.2%	82.6%	P<0.001	0.32	0.67

p*: McNemar's p-value **: p<0.001 †: n = 1547

Time used to process and read blood smears: We assessed the duration used to process and have BS results available from the day of sample collection at health facility, to district staining, reading, and upon reaching reference microscopists in Bagamoyo via Dar es Salaam. Table 5, presents a summary of results.

Table 5: Time (days) to process and obtain results from blood smears

Parameter	RDT to BS1	RDT to BS2	BS1 to BS2
Total tests	1708	1652	1544
Average (days)	7.7	43.9	36.4
Minimum (days)	1	19	16
Maximum (days)	33	98	90
25%	4	30	23
50%	7	45	37
75%	11	55	45

***Health workers understanding of mRDT policy and acceptance of RDT QA methods*:**

During a feedback session to the district and study facilities, we conducted an informal enquiry of health workers who use mRDTs, so as to assess health workers' understanding of the mRDT policy as well as the completed RDT QA study. A total of 30 health workers, 20 (67%) from Mufindi and 10 (33%) from Iringa participated. The characteristics of interviewed HWs are summarized in Table 6.

Table 6: Acceptance of RDT QA methods: description of health workers (HWs)

Characteristic of HWs	**Distribution**	**Frequency N= (%)**
Total HWs interviewed	All/ Female	30 (100)/ 20 (67)
HWs interviewed by district(s)	Mufindi DC	20 (67%)
Total number of staff in HF	Minimum/ Maximum	2/ 70
HWs by type of facilities	Hospital	10 (33)
	Health Center	15 (50)
HW by facility ownership	Government	25 (83)
	NGO (Religious)	5 (17)
HW cadre	Laboratory personnel	17 (57)
	Clinician	7 (23)
	Nurses/ Nurse Aide	6 (20)
Work experience	Above 10 years	13 (43)
	Between 3- 9 years	11 (37)
	Less than 3 years	6 (20)

Many health workers were aware that the use of mRDTs was due to changes in treatment policy (11/30), and patients who qualify for the test are those suspected to have malaria. Majority (16/30) related assessment of control line as a measure of test accuracy and suggested the use of microscopy for quality control of mRDT results. Health workers liked mRDT because they were rapid, easy to use and give reliable results. Their major concerns were mRDTs inability to give parasite count, stock-out of the tests kits in their working areas and its high rate of negative results.

Results of this qualitative enquiry are summarized in Table 7 below:

Table 7: Qualitative assessment of understanding and acceptance of mRDT and mRDT QA methods.

Characteristic variable/ indicator	Highest Freq (n)	Medium Freq (n)	Low Freq (n)	Other (n)
Why did the HF start using mRDT	Policy change (11)	Rapid results (10)	To confirm malaria (7)	Part of study (3)
What patients qualify for mRDT	Malaria symptoms (27)	Malaria suspects (3)	Symptoms, not used antimalaria (1)	All age (1)
How can you be sure results are correct	Control/ test lines (16)	Compare with BS (9)	Compare with clinical signs (7)	Non
Suggest how to verify tests results	Compare with BS (15)	Control line/ expiry/ lot (6)	Clinically including follow up (2)	Two readers (2)
What is good about mRDTs	Rapid (18)	Ease of use (12)	Reliable results (8)	Confirms malaria (3)
What is not good about mRDTs	No parasite count (4)	Too many negatives (3)	Different kits training/use (2)	Blood taking devices (1)
Advice about use of mRDT	Ensure timely availability (16)	Need more training (4)	Need quality control (4)	Use multi-specie tests (1)
Awareness of QA study (19/25) 76 - Preference between two methods:	BS because gives parasite count (13)	BS because it is easy to prepare and use (3)	DBS easy to take sample, detect low parasitemia (3)	Both BS and DBS to increase validity (2)

Health Workers Test Performance (Checklist)

We conducted 31 checklists to assess RDT stock, availability if RDT job-aides, information education and communication materials (IECs) related to mRDTs, waste disposal particularly sharps and steps of performing a test. Sometimes we were not able to observe any RDT testing procedure due to RDT stock-out or lack of patients sent for a test. Results presented in Table 8, show that all cadres related to clinical care of patients do perform mRDT testing but mostly laboratory personnel. Rarely a nurse was left in charge of determining a patient's treatment. RDT related job aides and IEC materials were almost non-existent, therefore rarely used. Assessment of buffer lot and expiry status was not common. Occasionally, health workers did not label used RDT cassettes, but placed them on respective patient's file before reading the result. Mobile phones were commonly used as time devices.

The overall performance of the testing ranged between moderate (>50% YES scores), to very good (>80% YES scores) for part B of a checklist, see Table 8 below.

Table 8: Checklist of RDT stock status and health worker performance

Code and activity checked	**Scores n (%)**			
A1: Who performs RDT (multiple responses)	Lab Asst=20	C/ Officer=06	Lab Tech=01	Nurse=8
A2: Who determines treatment (multiple response possible)	C/ Officer=23	AMO=05	Nurse=7	
	Present /Yes	**Absent /No**	**N/A**	**Total**
A3: RDT stock status	21 (67.7)	10 (32.3)	-	31
A4: RDT (good) storage conditions	27 (100)	0	-	27
A5: Presence of RDT job-aides	8 (26.7)	22 (73.3)	-	30
A6: Presence of RDT IECs	0	30 (100)	-	30
A7: Available containers for sharps disposal	27 (96.4)	1 (3.6)	-	28
	Yes	**No**	**Not checked**	**Total**
B1: Patient has fever	17 (68)	8 (32)	-	25
B2: HW uses job aides	5 (21)	19 (79)	-	24
B3: HW check expiry	9 (39.2)	14 (60.8)	-	23
B4: HW uses gloves	22 (95.7)	1 (4.43	-	23
B5: HW counsel the patient	18 (75)	6 (25)	-	24
B6: Drying of alcohol	20 (100)	0	-	20
B7: Blood drawing tech	19 (79.2)	5 (20.8)	-	24
B8: How blood is deposited	20 (87)	3 (13)	-	23
B9: Buffer drops	22 (100)	0	-	22
B10: Checking buffer lot/ expiry	313.6)	19 (86.4)	-	22
B11: Labeling	18 (78.3)	5* (21.7)	-	23
B12: Use of timer**	23 (100)	0	-	23
B13: Read results in 15 minutes	15 (75)	0	5 (25)	20
B14: Disposal of sharps	19 (90.5)	2 (9.5)	-	21
B15: Performance: Poor/ Average/ V-good	Poor=1	Moderate=10	V/good=10	21

* place unlabeled RDT on patient file ** use of mobile phones as timing devices

CHALLENGES:

There were several challenges encountered during the conduct of this work. Most of them were related to collection and processing of microscopy readings as well as molecular analysis of dried blood spots as listed below:

1. Poor quality of blood smears made at health facilities that do not have laboratory services e.g. dispensaries. This led to problems in staining and reading of smears.
2. Challenges with transportation of stained blood smears- 60 out of 1710 BS1 slides (3.5%) could not be reviewed with reference microscopists
3. In-ability to prepare P. falciparum DNA standard solutions as positive controls to be used for PCR analysis in Tanzania. They were made at the CDC laboratory in Atlanta, and transported to Bagamoyo.
    a. The last batch of DNA standard was received in September 16, 2011
4. Problems with a PCR machine and limited technical competence to repair PCR machine, which necessitated a shipment of the machine to Germany, to the manufacturer.
    a. The shipment for repair took place between (including dates when the machine was sent outside the country and brought back) March and April, 2010
    b. Technical repair from a technician from Anatech Technologies (South Africa): in February 15, 2011
5. Loss of data due to duplicate id sticker labels and incomplete information for some records (about 1.9%, i.e. 45 out of 2369 records did not have complete information and 487 results were not included in the final analysis because of duplicate identification). This highlights the challenges of sample identification if QA is not performed onsite.

CONCLUSIONS:

The prevalence of malaria in Iringa Region is low as has been previously reported in national surveys (THMIS, 2007/8). In this study malaria positivity was higher with mRDTs than microscopy and qPCR. The high rate of mRDT false positive results could be due to many factors that can be grouped into 2; First: Health workers' related factors such as 1) Incorrect reading of RDT results by health workers 2) Incorrect testing procedure during RDT testing 3) Substituting buffer solution with other liquid such as normal saline, distilled water, tap water or buffer from different lots/ batches, as has been reported by Gillet P, et al 2010 [9]. Second: other reasons for mRDTs false positivity may be related to the devices and/ or technology. These includes1) Persistent antigenemia (HRP2) following a successful treatment of malaria 2) Faulty within the test devices 3) Presence of circulating antinuclear antibodies such as in patients with Rheumatoid arthritis [10,11]. Also, a strict cut-off parasite density of a test to be considered as positive set by the study team may have influenced the observed mRDTs false positivity rate.

The difference in malaria prevalence between the two districts was apparent in this study. When qPCR was taken as a gold standard, the overall prevalence was very low, but much lower in Mufindi than Iringa DC. This low disease prevalence plays a significant role in diagnostic tests performance in terms of sensitivity specificity, disease predictive values [12]. As prevalence was higher with mRDTs than microscopy, this also translated into better mRDTs sensitivity than microscopy when qPCR was used as a gold standard. Both mRDTs and microscopy recorded very high specificity when compared against qPCR. However, the positive predictive value (PPV) was considerably lower with mRDT than microscopy. The disease predictive values (positive and negative) have direct impact in clinical practice because they provide an estimate of proportion of diseased people among those tested positive. In this study, microscopy provided a better estimation of diseased patients out of all positively tested individuals, than mRDTs when compared to qPCR results. This could also be explained by the higher false positive rate observed from mRDTs results.

Higher inter-observer agreement (as measured by kappa coefficient) was observed between microscopic analysis of blood smears between the districts laboratory technicians and reference laboratory technologists. This is a measure of observer's precision (good reliability) [13]. Comparison with qPCR demonstrated a better inter-observer agreement with microscopy than mRDTs. On the other hand, district and reference microscopists had a substantial higher inter-observer agreement than other test combinations (table 4). This observation may be closely related to the mechanisms that the tests use to

diagnose a malaria infection. Where mRDTs detects parasite antigens, microscope does a morphological assessment of a parasite and PCR detects parasite DNA from whole blood [14]. With these findings, the significance of using microscopy for diagnosis of malaria in clinical settings cannot be overly emphasized.

Assessment of the time needed to process BS at district level revealed that, smears at district level may take on average 8 days (min 2 to max 33) to be processed; but up to 44 days (min 19 to max 98) to get a second reading; especially if smears are to be transported to another region. Given the logistical and technical challenges encountered in this study, we were not able to systematically assess the time needed to process and get PCR results. However, the technical competence shown by district's microscopists to stain and read slides, despite the complaints of poor quality of smears from the facilities is encouraging. During the initiation of this study, key stakeholders (NMCP and PMI), asked the study team to utilize district's technicians/technologists who have undergone a microscopy competence training at the National Institute for Medical Research (NIMR), Tanzania, led by the Walter Reed Army Institute of Research, USA. These microscopists would have undergone competence training similar to what was described for establishing a center of excellence in Kisumu, Kenya [15]. The performance shown by the districts' technicians was very much comparable and correlated with the reference microscopists. This indicated a sufficient technical competence to act as quality assurance officers in their respective working districts.

Many health workers at participating district were aware of the new policy of malaria confirmation, and the kind of patients qualifying to get a test. Their major concerns were based on a fact that mRDTs does not give parasite count and not always available in health facilities. This concern was more apparent with the in-availability of the tests devices in the facilities (table 8). Assessment of the health workers testing performance reviled that, job-aids are seldom used. Performers are not accustomed to check device and / or buffer solution expiry dates. This observation shows the need to stress abiding to the testing standard operating procedures (SOP).

In summary, when comparing the two mRDT quality assurance methods assessed in this study, the following conclusions can be made: That, PCR can be suitable for routine mRDT QA assessment simply because it has less logistical demand for storage and transportation of samples; but it provides:

1. Technical challenges related to running and repair of machines, i.e. materials are machine specific and sometimes procurement needs expert opinions, storage and maintenance of reagents and aliquots is

resource demanding (e.g. maintaining cold chain) and problems with the acquisition of DNA standard solutions (of varying parasite concentration) for analysis of samples

2. Less correlated with RDT results: with higher inter-tests variability as measured by kappa statistic and test performance (sensitivity, PPV). This may be due to the high rate of false positives provided by mRDTs, but nevertheless it provides technical implication in the interpretation of findings and administrative measures to follow as a result of QA assessment

Microscopy is suitable for routine assessment of mRDT performance because:

1. Better correlation with RDT results in kappa coefficient and tests performance (sensitivity, PPV), hence easy to interpret and act upon the findings
2. Better correlation with PCR than was mRDT in the proportion of positive results; hence provides a more realistic measure of local malaria prevalence
3. Better correlation amongst district and reference readings
    a. Districts QA officers who have undergone competence training at NIMR are sufficient to carry out district based/ local assessment of mRDT performance
4. Less technical and logistics demand- depending on the level of assessment and movement of slides for reference reading
5. Cost is likely to be minimal and less time is needed to complete the assessment exercise

However, logistical challenges of BS transportation from one area to another may result in loss of samples and data.

As well, poor smears made by health workers in health facilities that do not have trained laboratory personnel's may complicate the evaluation process, as has been reported previously [8].

LIMITATIONS:

In the planning stage, this evaluation also aimed at assessing the cost and timeliness of the two RDT QA methods studied; but due to the challenges associated with processing of DBS, this assessment would have been influenced by additional measures used to solve these challenges and lead to biased assessment. Therefore, we were not able to carry out a detailed analysis to answer this objective.

Secondary to the problems related to molecular analysis of malaria parasites, a protocol was amended to provide a second measure of qPCR assessment, but so far the efforts have not been fully effective as the samples transfer to the CDC laboratory were delayed; mainly due to delays in sample processing (DNA extraction) at Bagamoyo laboratory. As well, when we did send some DNA extracts aliquots, sometimes the solution was not sufficient to carry out appropriate analysis. This was complicated by the transportation process of the aliquots ampoules to Atlanta. As a result, assessment of the second qPCR was challenged and could not provide an exhaustive assessment of all parameters reported.

The quantitative cut-off point for a test to be regarded as positive (between qPCR and microscopy) may have some influence in mRDTs performance and lead to increased false positivity.

RECOMMENDATIONS:

Challenges encountered in this study, have highlighted the consequences of using PCR analysis for monitoring performance of mRDT in routine use. The technical challenges to maintain the PCR machine (as was the case in this evaluation) as well as demands for machine specific equipment and reagents makes it unsuitable for routine national-wide mRDT quality assessment.

However, the competence shown by laboratory technicians/ technologists who have undergone a microscopy competence training at NIMR- as a special programme run by Walter Reed/ PMI/ NIMR, etc, is enough to perform district based and regional or zonal-wide quality assurance of mRDTs in routine use. The need for routine refresher training of the competent microscopists' is highly recommended.

During the implementation of microscope-based mRDT QA, long distance transportation of the smears for routine assessment should be avoided; except periodically with reliable transport and manpower in place.

The potential of using used RDT devices as a source of parasite DNA for molecular analysis to monitor mRDT performance in field [16] should be further explored. In the field, mRDTs false positivity and prozone effect [17] need to be thoroughly monitored to facilitate clinical interpretation of tests result.

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Below is a summary of the project's expenditure:

Report for RDT Project for the period ending September, 2011

Line Item No.	Budget Line Item	Total Approved Budget(USD)	Total Approved Budget(TZS)	Cumulative To date (TZS)	Balance Remaining (TZS)	% Spent
1	Salary & Benefits	89,200.00	107,040,000.00	130,544,764.46	(23,504,764.46)	122%
2	Equipment	-	-	-	-	0%
3	Supplies	22,420.00	26,904,000.00	60,978,095.55	(34,074,095.55)	227%
4	Travel	41,450	49,740,000.00	29,233,360.84	20,506,639.16	59%
5	Others	11,930.00	14,316,000.00	18,668,834.70	(4,352,834.70)	130%
	Total	**165,000.00**	**198,000,000.00**	**239,425,055.55**	(41,425,055.55)	121%

A detailed financial report can be made available from the institute's finance department.

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APPENDICES:

Appendix 1: Questionnaire used to assess health worker's acceptability of RDTs QA method- attached as a separate file

Appendix 3: Checklist used to assess health workers testing performance

IFAKARA HEALTH INSTITUTE

Malaria Rapid Diagnostic Tests Quality Assurance Programme

SUPERVISION CHECKLIST *for RDTs Stock and health workers' Performance*

District name:_____

Facility name:_____ *Checklist no: _____*

Part A: RDT Patient Flow, Stock, and Storage Condition:

CODE	***Things to be checked and discussed***	***No Problem***	***Identify problem***	***Remarks***
A/01	*Who performs the RDT?*			
A/02	*Who determines treatment given?*			
A/03	*Status of the stock; RDTs boxes/ kits in stock (#)*			
A/04	*Storage condition of RDTs adequate:*			
	:Temperature (record)			
	:Wall, Roof leakage			
	:Security conditions			
A/05	*mRDTs Job aid present at testing site*			
A/06	*Any mRDTs IEC materials present (#)*			
A/07	*Available disposal of sharps and used RDT kits*			

Part B: RDTs Performance:

CODE	***Things to be checked and discussed***	***Patient 1***	***Patient 2***	***Remarks***
B/01	*Does the patient being tested have fever?*			
B/02	*Check if HCW uses RDT job aid*			
B/03	*Check if HCW observes the expiration date on packaging and dessicant before performing the test*			
B/04	*Check if HCW wears gloves to perform RDTest*			
B/05	*Check if HCW explains the testing procedure to the patient*			
B/06	*Check if the alcohol is allowed to dry before pricking*			
B/07	*Check how and amount of blood collected for test*			
B/08	*Check how blood is deposited in appropriate hole*			
B/09	*Check how buffer drops are kept in appropriate hole*			
B/10	*Check buffer lot number and expiration date*			
B/11	*Check if the test kit is clearly labeled*			
B/12	*Check if the HCW has and uses a timer*			
B/13	*Check if results are read after 15 minutes*			
B/14	*Check if lancet and used tube are disposed properly*			
Comment on performance	*:Poor (<7/13)*			
	:Adequate (8-10/13)			
	:Very good (11-13/13)			

Part C: Training of HCW:

CODE	***Things to be checked and discussed***	***Yes/No***	***If Yes; by whom***	***Remarks***
C/01	*Was a performing heath worker trained how to use mRDT*			
C/02	*Number of HCW trained mRDT use in this HF*			

Supervision done by:

1:Name:_____ Title:_____

- END OF REPORT -