Malaria prevalence and performance of diagnostic tests among hospitalized fever patients in Zanzibar

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Authors

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Abstract

Background: Control efforts in Zanzibar reduced the burden of malaria substantially from 2000 to 2015, but re-emergence of falciparum malaria has been observed the last years. This study evaluated the prevalence of malaria and performance of routine diagnostic tests among hospitalized fever patients in a 1.5 years period in 2015 and 2016.

Methods: From March 2015 to October 2016, pediatric and adult patients hospitalized with acute undifferentiated fever at Mnazi Mmoja Hospital, Zanzibar were included. The malaria prevalence was assessed by polymerase chain reaction (PCR), rapid diagnostic test (RDT) and routine microscopy.

Results: Malaria prevalence was 8% (67/820). All cases identified by PCR had *Plasmodium falciparum* infection, except for one *P. ovale*. Compared to PCR, the RDT had sensitivity of 64% (36/56), specificity 98% (561/575), positive predictive value (PPV) 72% (36/50) and negative predictive value (NPV) 97% (561/581). Microscopy had a sensitivity of 50% (18/36), specificity 99% (251/254), PPV 86% (18/21) and NPV 93% (251/269) compared to PCR.

Conclusions: A high malaria prevalence was identified compared to previous studies in Zanzibar. Microscopy showed higher PPV than RDT in this study. Both RDT and microscopy had low sensitivity compared to PCR. However, low parasitemia identified only by PCR in a semi-immune individual could be coincidental and may not be the cause of the presenting symptoms. To achieve malaria elimination in Zanzibar, PCR-based surveillance is suitable to guide control and elimination efforts.
Keywords: Malaria, prevalence, surveillance, microscopy, point-of-care diagnostic tests, polymerase chain reaction, Zanzibar, Tanzania, Eastern Africa

Introduction

Successful control efforts reduced the burden of malaria in Zanzibar substantially from 2000 to 2015 (1). However, this progress has halted in recent years. According to the World Health Organization (WHO), Sub-Saharan Africa suffered 384,000 estimated malaria deaths in 2020, equaling 94% of the global malaria death toll (2, 3). In the Zanzibar archipelago, a comprehensive control and elimination program was implemented in 2001, introducing artemisinin-combination therapy, intermittent treatment in pregnancy, nationwide distribution of long-lasting insecticide-treated bednets, indoor residual spraying, active case detection among contacts and larvicidal treatment of mosquito breeding sites (4). The interventions reduced malaria-prevalence by 96% from 2002 to 2015 (5), malaria in-patient cases by 78% from 1999 to 2008 (6), and cut reported deaths to negligible. However, since 2016 the number of reported cases in Zanzibar has increased (2), and in 2020, the Zanzibar Ministry of Health intensified control measures after a surge in malaria cases during a prolonged rainy season.

Commercially available malaria rapid diagnostic tests (RDTs) differ widely in sensitivity and specificity (7), and accurate microscopy depends on high quality technical equipment and experience. PCR has high sensitivity and detect parasitemia lower than 1 p/µL, while the detection limits for microscopy and sensitive RDTs are around 50-200 p/µL and 100 p/µL, respectively (8).

The main objective of this study was to evaluate the prevalence of malaria identified by routine tests and PCR, and the performance of RDT and microscopy in assessing febrile patients admitted to Mnazi Mmoja Hospital (MMH), Zanzibar.

Methods
Patient material

From 17th March 2015 to 4th October 2016, we consecutively enrolled patients with acute undifferentiated febrile illness admitted to the Department of Internal Medicine and the Department of Pediatrics at MMH. With 544 beds, this hospital in Zanzibar city is the referral hospital for the 1.3 million population of the Zanzibar Archipelago (9). Inclusion criteria were fever (≥38.3°C in adults, ≥38.5°C in children) or hypothermia (<36.0°C), tachypnea >20/min, tachycardia >90/min on admission or attending clinicians’ diagnosis of severe acute infection. Neonates under 15 days of age were excluded. Demographic and clinical information was obtained using a standardized case-report form.

We obtained blood for on-site RDT and microscopy, and blood in EDTA tubes stored at -20°C and shipped on dry ice to Norway for malaria-PCR to be done later. Malaria microscopy was performed if requested by attending clinician, while PCR and RDT was performed on all patients for the sake of the study.

A positive result from one or more of the three tests was used to determine the prevalence of malaria. In case of discrepancy between routine diagnostics and PCR, PCR results were defined as final results. In case of discrepancy and PCR was missing, a positive result from RDT or microscopy was defined as a malaria case. In order to evaluate the performance of routine diagnostic tests compared to a more sensitive and specific reference method, results from microscopy and RDT were compared to PCR.

Microscopy and rapid diagnostic test

For microscopy, a Giemsa stain (10% solution) of both thick and thin blood film was performed in accordance with hospital procedures.

The RDT First Response Malaria Ag pLDH/HRP2 Combo Card Test (Premier Medical Corporation Ltd., India) was used until 20.08.2016. At this time, for the remaining six weeks,
it was replaced by CareStartTM Malaria HRP2/pLDH (Pf/PAN) Combo (Access Bio, Inc., Somerset, NJ, USA) due to stock-out. Ninety-six percent (685/714) of the patients were tested with the first RDT.

**PCR methods**

DNA was extracted from 500 µl whole blood using MagNA Pure 96 DNA and Viral NA Large Volume Kit (Roche Diagnostics GmbH, Mannheim, Germany). Presence of *Plasmodium* DNA was assessed applying a genus-specific PCR, and quantitative analysis (q-PCR) was performed using a customized plasmid (10). For quality assurance, results with cycle threshold values ≥30 were re-analyzed in triplicates, and in case of discordant results between PCR, RDT or routine microcopy, samples were retested from DNA extraction, using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Genus-specific PCR positive samples were re-analyzed by species-specific real-time PCR assays targeting the 18S gene of *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. Previously published primers (11, 12) were applied in separate master mixes with the following concentrations: 200 nM for *P. falciparum*, 100 nM for *P. vivax*, 300 nM for *P. ovale*, and 200 nM for *P. malariae*. Each reaction mixture also contained 2 µl template, and 12.5 µl SYBR Select Master Mix (Applied Biosystems, Carlsbad, CA, USA), at a total volume of 25 µl. Amplifications were performed using the following cycling parameters: Step 1, 50°C for 2 min; step 2, 95°C for 10 min; step 3, denaturation at 95°C for 15 sec and step 4, annealing at 60°C for 1 min; steps 3-4 repeated 40 times. Genus-specific positives not detected by species-specific PCR were sequenced to identify species (10, 11).

**Statistics**

Dichotomous variables were assessed by Chi-square test, and by binomial logistic regression for factors with multiple levels (i.e. age groups). Continuous variables such as age and
parasitemia, were assessed by Wilcoxon rank-sum test. Analyses were performed in R version 4.0.4, Rstudio version 1.4.1103 (R Core Team, Vienna, Austria) (13). Figure 2 and 3 were made using the ggplot2 package in R.

Results
Among 1044 patients fulfilling the inclusion criteria, we excluded 207 neonates and 17 patients from whom we could not obtain blood for testing, resulting in a study population of 820 (Figure 1). Fifty-seven percent (470/820) were males. Median age was 13 years, range 16 days – 95 years. Forty-nine percent were admitted to the Department of Pediatrics (n=404, age range 16 days – 14 years) and 51% to the Department of Internal Medicine (n=416, age range 13 – 95 years).

Malaria prevalence was 8% (67/820). All positive cases had *Plasmodium falciparum* infection, except for one, who had *P. ovale* identified by sequencing (parasitemia 1.1x10⁵ copies/µl blood, negative RDT, positive microscopy). Malaria PCR was positive in 63/731 (9%) of patients tested, and RDT in 54/714 (8%). Compared to PCR, the RDT had a sensitivity of 64% (36/56), specificity 98% (561/575), positive predictive value (PPV) 72% (36/50) and negative predictive value (NPV) 97% (561/581). Malaria microscopy was performed for 40% (325/820) of whom 7% (n=22) were positive. Microscopy had a sensitivity of 50% (18/36) and a specificity of 99% (251/254), PPV 86% (18/21) and NPV 93% (251/269) compared to PCR (Table 1).

Among patients with PCR-confirmed malaria, the parasitemia was higher in those with positive microscopy (median 2x10⁵ copies per µL) than those with negative result (median 9x10³ copies/µL, p=0.006), and parasitemia was higher in those with positive RDT (median 2x10⁵ vs median 7x10³ copies/µL, p=0.002, Figure 2).
Malaria prevalence was 10% (45/470) in males and 6% (22/350) in females, however, this difference was not statistically significant (OR 1.6, CI 0.9-2.7, p= 0.1). There was significantly higher malaria prevalence in age groups 1-4 years (8%, OR 4.0, CI 1.2-17.8), 5-9 years (14%, OR 7.9, CI 2.3-34.6), 10-19 years (17%, OR 9.4, 3.1-40.7) and 20-29 years (11%, OR 5.5, CI 1.5-25.9), compared to children under 1 year old as reference (2%, Figure 3).

Travel history was recorded for 33% (273/820). Malaria patients were more likely to have travelled to mainland Tanzania within the past six months (41%, 12/29), than those testing negative (4%, 10/244, OR 16.5, CI 6.2-43.7, p<0.001). Ten of the 22 malaria patients with known travel history had not visited the mainland, four of these had negative RDT. However, all ten cases of presumed autochthonous malaria were positive on PCR with a median parasitemia of 5.0x10^4 copies/µL blood (range 0.15 to 6.0x10^5).

Monthly variations in malaria prevalence are shown in figure 4. An increase of malaria cases was observed at the end and shortly after the rainy season.

**Discussion**

In this study on patients hospitalized for acute febrile illness, a high malaria prevalence was identified by PCR and routine malaria diagnostic tests compared to previous reports from Zanzibar. In a survey in 2009 to 2010 at three hospitals in Pemba, the second largest island of the Zanzibar archipelago, 1% of febrile patients were positive for malaria with RDT and/or microscopy (14).

There are no previous PCR-based malaria prevalence studies in hospitalized patients in Zanzibar. Previous community-based studies in Zanzibar report a prevalence below 3% up to 2015, including PCR-based studies (15-17). In 2015, a PCR-based study documented a 2% malaria prevalence in out-patients from rural areas of the two main islands of Zanzibar (5).
In line with previous studies (15), malaria prevalence in the present study was higher in children, teenagers and young adults. This may possibly reflect semi-immunity in older individuals exposed to malaria prior to implementation of the comprehensive malaria control program.

Malaria was strongly associated with travel to mainland Tanzania within the past six months. This is also shown in recent molecular studies substantiating malaria import from the mainland (18). However, ten patients had no travel history, confirming autochthonous malaria transmission inside the Zanzibar archipelago. Four of these had negative RDT and would have been missed by routine diagnostics. In a survey of out-patients from rural areas of the two main islands of Zanzibar in the period from 2003 to 2015, findings imply ongoing autochthonous transmission (5).

Our finding of rising malaria prevalence shortly after rainy periods is in line with the study from Zanzibar (5) and numerous other studies. The present study indicates that malaria had resurged as an important cause of febrile illness in Zanzibar by 2015. While fifteen years of comprehensive malaria control greatly reduced malaria incidence in the archipelago, it also rendered children and teenagers with less immunity and increased susceptible to malaria. Our finding of high malaria prevalence in children and teenagers underlines the risk of severe malaria in a non-immune population. The study also highlights the risk of importation of malaria from near-by mainland Tanzania, and documents ongoing autochthonous transmission in Zanzibar. Considering the presence of the effective malaria vector *Anopheles gambiae*, and increasing resistance of vectors to pyrethroid (19), re-introduced malaria can spread quickly in the population.

The RDT showed a slightly poorer performance compared to PCR than reported previously from Zanzibar (15). The lower PPV of RDTs in the present study (72%) compared to 97% in the study of Shakely et al. (15) may be explained by an inferior performance of the RDTs. In
a WHO evaluation, the sensitivity for detecting 200 *P. falciparum* p/µL was scored 85% and 90% for the tests used in the present study (RDT First Response Malaria Ag. pLDH/HRP2 Combo Card Test and CareStartTM Malaria HRP2/pLDH (Pf/PAN) Combo Test). In comparison, the Paracheck Pf Test (Orchid Biomedical Systems, Goa, India) used in the previous study (15) had a sensitivity of 95.9% (20). The false-positivity rate of the three tests was 0.0%, 0.4% and 1.3%, respectively. However, the high NPV indicates that using RDT or microscopy is relatively safe since less than 4% (RDT) and 7% (microscopy) of malaria cases were missed. Since PCR has very high sensitivity, it is possible that some of the discrepancy between PCR and RDT/microscopy could be due to coincidental non-significant low-level parasitemia in semi-immune individuals suffering from febrile illness of other causes. Indeed, patients positive only by PCR had significantly lower parasitemia than those who also had positive RDT and/or microscopy (Figure 2).

The superior sensitivity of PCR compared to microscopy is well known (8), and may, apart from inherent methodological issues, be due to suboptimal staining of blood slides, malfunctioning microscopes and deficient training of the laboratory technician (21). In the present study, sensitivity of microscopy is still substantially higher than in several other surveys (22). Our findings are in line with a review comparing PCR and microscopy for malaria diagnosis in endemic areas, which found that PCR identified on average twice the number of malaria infections compared to microscopy (23). While PCR is highly sensitive, the level of parasitemia detected by RDT and microscopy corresponds well with clinically relevant malaria (24). Our study could not evaluate whether low-level parasitemia was associated with other causes of fever. With its documented high sensitivity, PCR has an obvious role in malaria surveillance. However, for clinical diagnosis of acute undifferentiated febrile illness, PCR has
disadvantages of high cost and technical requirements on laboratories, as well as the potential
for detecting non-significant low level malaria parasitemia, and detecting DNA remains of
non-viable parasites several weeks after parasite clearance (25). In a study from 2015, PCR
was positive in 2% of asymptomatic individuals in Zanzibar (26). PCR’s excellent ability to
detect low-level parasitemia makes this method particularly suitable for surveillance in
settings of near-elimination of malaria, such as in Zanzibar (27).

Conclusions

The study reveals a high malaria prevalence in febrile patients admitted to MMH, and
confirms autochthonous malaria-transmission in Zanzibar. RDTs and microscopy reliably
detect high malaria parasitemia. The superior sensitivity of PCR as compared to routine
diagnostics supports its suitability for guiding control and elimination efforts in Zanzibar.

List of abbreviations

MMH: Mnazi Mmoja Hospital

NPV: Negative predictive value

PCR: Polymerase chain reaction

PPV: Positive predictive value

RDT: Rapid diagnostic test

Declarations

Ethical approval and consent to participate

The research protocol was approved by the Zanzibar Medical Research and Ethical
Committee (record no ZAMREC/0002/November/2014, renewal no
ZAHREC/02/June/2019/41), and by the Regional Committee for Medical Research Ethics
Health Region South East Norway (record no 2014/1940/REK South-East). Inclusion was subject to informed consent from patient or custodian.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

AO, BB, KM and CGH conceived the study. AO, M. Miraji, KAA, KOK, MR and KM recruited patients and collected data for the study. CGH and KOK performed laboratory analyses. AO, BB and CGH analyzed the data. BB and AO wrote the first draft of the manuscript. All authors contributed to discussion of the data and revision of the manuscript. All authors read and approved the final manuscript.

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Authors’ information

Not applicable.

Footnotes

Not applicable.
Figure 1. Patients included and analyses performed

Figure 2. Quantitation of parasitemia by real-time PCR by diagnostic modalities expressed as log-transformed values of copies per µL blood. Dots represent individual observations. Number tested in brackets.

Micro+, microscopy positive; Micro-, microscopy negative; RDT+, rapid diagnostic test positive; RDT-, rapid diagnostic test negative.
Figure 3. Number of malaria patients (blue bars) among 820 febrile patients (grey bars) and percentage positive (line) in different age groups.

Fig 4. Monthly number of malaria cases and rainfall

- Number malaria positive
- Monthly rainfall (mm)
Figure 4. Monthly number of malaria cases and monthly rainfall from February 2015 to October 2016. Rainfall data for Dar es Salaam from the Tanzanian Meteorological Agency (TMA) (28, 29). Study start March 17, 2015, study end October 4, 2016.

Table 1: Performance of RDT and microscopy compared to PCR among patients hospitalized with fever in Zanzibar (total n=820)

|                    | RDT (n=631) | Microscopy (n=290) |
|--------------------|-------------|--------------------|
|                    | Percentage (n/total) | Percentage (n/total) |
| Sensitivity        | 64% (36/56) | 50% (18/36)        |
| Specificity        | 98% (561/575) | 99% (251/254)      |
| Positive predictive value | 72% (36/50) | 86% (18/21)        |
| Negative predictive value | 97% (561/581) | 93% (251/269)      |

Numbers given for patients investigated with PCR and each test. Discrepancies are due to missing values.

Abbreviations: RDT, rapid diagnostic test; PCR, polymerase chain reaction.

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