

Diagnosing pediatric malaria in Dar es Salaam, Tanzania

Clinical and laboratorial perspectives

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Scientific environment

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Summary

Malaria is an extensive global health issue with high morbidity and mortality. Diagnostics of malaria are challenging. Clinically malaria is indistinguishable from various other infectious diseases. Microscopy of blood smears has generally been considered the gold standard for malaria diagnostics. Rapid diagnostic tests (RDTs) and PCR are being used increasingly and may prove to be superior alternatives to microscopy, which has many limitations. However, microscopy will likely never be entirely replaced by any other method, as it is ideal for species identification, quantifying parasitemia and following response to treatment.

In this study routine and research malaria microscopy, PCR and RDTs for malaria diagnostics were compared. PCR detected the most cases. Positive RDT was associated with symptoms of severe malaria. Routine malaria microscopy performed poorly and was only confirmed in 43-53% of cases depending on choice of reference method. A strong association was found between anemia and thrombocytopenia and positive malaria results. In addition, palmar pallor and evaluation of severity of palmar pallor corresponded well with hemoglobin level.

No asymptomatic malaria was found among 108 young children recruited at a health clinic in Dar es Salaam when tested with RDT and PCR of DNA extracted from dried blood spots on filter paper (DBS).

Several DNA extraction methods from DBS were tested on a dilution series of a standardized *Plasmodium* positive sample and most performed similarly. However, the method using Chelex-100® and soaking of DBS punches in saponin overnight proved to be the method with the lowest limit of detection without missing any dilutions. This is also a method that has been used in many other studies. A TE-buffer based method performed much poorer than expected based on a previous study.

PCR done on DBS detected only approximately 54% of cases positive by whole blood PCR. This was much lower than in previous studies. All cases positive on PCR of DNA extracted from DBS were also positive by RDT and by *Plasmodium falciparum* species-specific PCR. This confirms that it is likely that PCR of DBS detected the cases with highest parasitemia.

The results of these studies support the use of RDTs for routine diagnostics where this is possible and when it is unnecessary to follow the effect of treatment or consider recrudescence, as routine microscopy performed poorly. Further studies are recommended to study asymptomatic malaria in a broader age group and a larger study population. Also, more studies comparing PCR of DNA extracted from DBS and whole blood should be done to assess whether the results of this study are representative. DBS have become popular to use in research settings in rural areas and where transport is difficult and should be evaluated to determine whether they truly perform as poorly as this study showed. Using Chelex-100® along with soaking in saponin overnight appears to be a good method for extracting DNA from DBS.

List of papers

The papers are referred to by their roman numerals in later references to them.

- Paper I: Strom GE, Haanshuus CG, Fataki M, Langeland N, Blomberg B: **Challenges in diagnosing paediatric malaria in Dar es Salaam, Tanzania.** *Malar J* 2013, **12**:228.
- Paper II: Strom GE, Tellevik M, Fataki M, Langeland N, Blomberg B: **No asymptomatic malaria parasitaemia found among 108 young children at one health facility in Dar es Salaam, Tanzania.** *Malar J* 2013, **12**:417
- Paper III: Strom GE, Tellevik MG, Hanevik K, Langeland N, Blomberg B: **Comparing four methods for extracting DNA from dried blood on filter paper for PCR targeting mitochondrial *Plasmodium* genome.** Submitted
- Paper IV: Strom GE, Moyo S, Fataki M, Langeland N, Blomberg B. **PCR targeting *Plasmodium* mitochondrial genome of DNA extracted from dried blood on filter paper compared to whole blood.** Submitted

Abbreviations

ACT	Artemisinin combination therapy
COSTECH	Tanzania Commission for Science and Technology
DBS	Dried blood spot
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
HRP2	Histidine-rich protein 2
LAMP	Loop-mediated isothermal amplification
MCHC	Maternal and child health clinic
MNH	Muhimbili National Hospital
MUHAS	Muhimbili University of Health and Allied Sciences
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pLDH	<i>Plasmodium</i> lactate dehydrogenase
RBC	Red blood cell
RDT	Rapid diagnostic test
WBC	White blood cell
WHO	World Health Organization

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1. BACKGROUND

1.1 Malaria epidemiology

Malaria was endemic in 99 countries in 2010 [1], most of these being low-income countries. It was estimated that approximately 3.3 billion people were at risk of malaria in 2011 and in 2010 an estimated 660,000 to 1.2 million people died from malaria [2, 3]. Eighty percent of malaria cases and ninety percent of malaria deaths are estimated to occur in Africa with young children and pregnant women being the most severely affected [2].

The dominant *Plasmodium* species in East-Africa is *Plasmodium falciparum* [4]. *Plasmodium vivax* transmission is widely distributed globally, and dominates in highly populated malarious areas outside Africa [5-7]. *P. falciparum* is considered to be the most deadly of the five malaria species [8] (*P. falciparum*, *P. vivax*, *Plasmodium ovale*, *Plasmodium malariae*, *Plasmodium knowlesi*) though infections with *P. vivax* and *P. knowlesi* also can be fatal with various severe manifestations [7, 9-11]. *P. knowlesi* and *P. malariae* are difficult to distinguish microscopically, however *P. knowlesi* generally has more serious presentation and can be fatal as opposed to the benign *P. malariae* infection [11]. The case fatality rate of *P. knowlesi* infection as reported in a review by Antinori *et al* was 3.4% [12] while *P. falciparum* has a case fatality rate of up to nearly 15% in severe malaria cases depending on how promptly treatment is given and the treatment's effectiveness [8]. An estimated 98% of malaria cases in Africa were due to *P. falciparum* in 2010 [2] and the disease causes extensive morbidity worldwide.

Malaria is a vector-borne disease that is transferred by certain anopheline species of mosquitoes [8]. One of the greatest challenges in malaria elimination is vector control. The number of malaria cases and deaths have been falling the past decade [3, 4] and between 1945

and 2010 seventy-nine countries eliminated malaria [1]. This was likely due to increased distribution and use of insecticide-treated nets and indoor residual spraying, along with more precise diagnostics and treatment with effective and appropriate antimalarials as a result of increased malaria-funding globally and in the most affected nations. Malaria remains a large global health concern despite current and previous eradication efforts. Limitations that have contributed to counteract eradication efforts include resistance to insecticides and antimalarial drugs and limitations in funding as well as lacking environmental and economic development in affected countries. Efforts to eliminate malaria can cause a large reduction in malaria cases and deaths, but if the pressure of the efforts is reduced when the burden of malaria decreases this may lead to a resurgence of the disease [13].

Older antimalarials are no longer in use in many areas because of resistance. The effective recommended antimalarial treatment with artemisinin combination therapy (ACT) has become somewhat threatened by reports of developing resistance to artemisinin compounds in the Cambodia-Thailand area [14, 15]. Resistance to artemisinin has not yet been reported in sub-Saharan Africa but with resistance found in Asia, precise diagnostics and treatment are critical to preserve its effectiveness in other areas. The newer antimalarials are more expensive than previous traditional antimalarials such as chloroquine. The high cost of treatment further underlines the importance of precise diagnostics and less presumptive treatment of unproven malaria to avoid the large expense of overtreatment [16, 17].

1.2 Diagnostic methods

Precise malaria diagnostics are important to avoid misdiagnosis of malaria, which can result in lacking treatment of an alternative cause of symptoms, or alternatively overdiagnosis and -treatment of malaria contributing to developing antimalarial resistance. Various methods are

discussed below. A newer method, called loop-mediated isothermal amplification (LAMP), was not tested in these studies but recent studies have suggested it may be a promising method for performing molecular diagnostics under field conditions. Compared to PCR, which is the more frequently used molecular method, it is a simpler, less expensive and time-consuming method with lower risk of contamination [18].

1.2.1 Clinical diagnosis

Diagnostics of malaria based on clinical presentation alone, without parasitological confirmation, is common practice to identify malaria cases especially in areas with high transmission [19]. However, clinically malaria presents elusively. The main symptom, fever, is common in a multitude of infectious diseases and other malaria symptoms such as vomiting, diarrhea, malaise and tachycardia are also common in other diseases [5, 20, 21]. Several other serious infections, including sepsis, typhoid fever, rickettsioses, louse- and tick-borne relapsing fevers, dengue and other arbovirus infections mimic the presentation of severe malaria but require fundamentally different treatment. Previously the WHO recommended treating all febrile children in malaria-endemic areas, with no other apparent cause of fever than malaria, with antimalarials without parasitological confirmation [22]. However, empirical treatment on mere clinical assessment results in vast overtreatment due to overdiagnosis of malaria [16, 19]. A study done in Uganda by Opoka *et al* showed that there was increased mortality among patients who received a clinical diagnosis of malaria as opposed to those with blood microscopy confirmed malaria [23]. From 2010 WHO changed their recommendation to that all cases of malaria be confirmed by a parasitological diagnosis before treatment, if possible [24]. As a result of this changed WHO-recommendation there has been a recent increase in the use of rapid diagnostic tests (RDTs) for malaria, especially in the African region [2, 20].

Simple blood tests such as hemoglobin and thrombocyte count are useful to support clinical suspicion of malaria with both anemia and thrombocytopenia being common in malaria cases [25, 26]. Anemia is a common complication of malaria as a result of increased destruction of red blood cells (RBCs) and decreased RBC production and is often a sensitive, though much less specific, indicator of malaria in a febrile child in a malaria-endemic area [27, 28]. Many explanations of the pathogenesis of thrombocytopenia during malarial infection have been proposed and it is likely a complex process [26]. The combination of anemia and thrombocytopenia has proved to be fairly sensitive (80%) and specific (84%) for predicting malaria [25, 27].

1.2.2 Blood smear microscopy

Malaria was identified in a microscope for the first time by a French physician, Alphonse Laveran, in Algeria in 1880 [29]. The traditional diagnostic method for malaria has been light microscopy of Giemsa-stained blood smears since the early 1900s [30] and blood smear microscopy has long been considered the gold-standard for malaria diagnosis [31].

Microscopy has many advantages over other diagnostic methods, as it allows for species identification and quantification of parasitemia. As opposed to RDTs and PCR, microscopy can identify recrudescence and early reinfections. Microscopy is also useful to monitor the reduction of parasitemia quantitatively during treatment and is thus important in diagnostics and follow-up. There are disadvantages of using blood smear microscopy; artifacts from the staining solutions or from contaminants, as well as thrombocytes can be mistaken for being malaria parasites [32]. In a busy and low-resource clinical setting with poor equipment and sometimes insufficiently trained lab personnel [33, 34], malaria is often highly over- and misdiagnosed by microscopy [34, 35]. Underdiagnosis is also common as only a small

volume of blood is viewed during malaria microscopy and low parasitemia may therefore be overlooked resulting in false negatives [30, 36]. In addition, differentiating malaria species and identifying mixed infections can be difficult by microscopy [37-39]. The expected threshold for detection of malaria parasitemia using thick blood smear microscopy is approximately 10-50 p/µl [31, 40] or higher than 100 p/µl under field conditions [30, 31, 41]. Highly skilled and experienced technicians may detect levels of parasitemia as low as 5-10 p/µl [41]. With its many limitations the role of blood smear microscopy as a gold standard in malaria diagnostics is being called into question [42-44].

1.2.3 Rapid diagnostic tests (RDTs)

RDTs are being used increasingly and have been implemented in many countries. They are recommended by the WHO as routine diagnostics for malaria in areas where microscopy is unavailable or unreliable [24]. In Tanzania, studies have shown that the use of RDTs reduces overdiagnosis and overtreatment of malaria without any negative consequences for febrile RDT-negative patients that do not receive antimalarial treatment [21, 45, 46]. RDTs are quick and simple to perform, easy to interpret and not as prone to human-error as many other diagnostic methods. Their main limitation is that reinfection or recrudescence of infection is difficult to identify, as RDTs detecting histidine-rich protein 2 (HRP2) can remain positive for more than 14 to 56 days after completed treatment [47-49]. Especially in areas with moderate to high endemicity this may pose a diagnostic challenge [50]. The sensitivity and specificity and reliability of results of malaria RDTs varies greatly between products and manufacturers [51]. RDTs generally have a lowest limit of detection of approximately 100 p/µl [42].

Antigens that can be detected by RDTs are HRP2, *Plasmodium* lactate dehydrogenase (pLDH), and aldolase. Various combinations may be identified through separate antibody bands on a single RDT. HRP2 is a *P. falciparum*-specific antigen, though *P. falciparum* parasites in certain areas do not express HRP2 [52], while tests detecting pLDH can either detect all species (PAN; *P. falciparum*, *P. ovale*, *P. vivax*, and *P. malariae*) or be species-specific for *P. vivax* or *P. falciparum* [53]. The antigen pLDH is cleared approximately at the same time as the parasites are cleared from the blood and thus corresponds better with clinical infection than HRP2, which persists long after treatment. However, RDTs detecting pLDH are generally less sensitive than those detecting HRP2 [54] though studies vary in their conclusions [55]. RDTs detecting aldolase have proven to have very low sensitivity [56]. All three antigens are also expressed by gametocytes, which may persist post-treatment [53].

1.2.4 Polymerase chain reaction (PCR)

PCR has generally only been used for research purposes. It has been attempted implemented for routine malaria diagnostics of imported cases in a European hospital over a shorter period of time, but the attempt did not result in a permanent change of first-line routine malaria diagnostics [57]. The greatest advantage of PCR is its high sensitivity and specificity [44, 58, 59]. It is, however, an time-consuming method requiring specialized expertise and expensive equipment to perform [60]. A PCR assay of one sample may cost up to approximately 6-9 USD depending on the methods used for extraction of DNA and for PCR [61] compared to 0.82-4.93 USD for a RDT depending on the manufacturer and type of RDT [62, 63]. Optimization of PCR assays and newer PCR techniques with simpler DNA extraction and analysis of PCR products may in the future make PCR feasible for routine malaria diagnostics in high resource settings [64].

Traditionally, DNA template for use in PCR reactions for *Plasmodium* species has been extracted from whole blood. However, dried blood on filter paper (dried blood spot, DBS) has been used increasingly in recent years [65-71]. PCR is a robust technique and DNA from properly stored and transported material can be extracted many years after collection for PCR analysis [40].

1.2.4.1 Dried blood spots (DBS)

While various methods have been employed for extracting DNA from DBS few studies have been done comparing different DNA extraction methods [72, 73]. Most of the studies that have been done on extraction methods include few methods tested on unstandardized samples [68, 74, 75]. Studies have shown some discrepancies in results between whole blood and DBS varying from very little or nothing to a 10- to 100-fold decrease in sensitivity when using DBS rather than whole blood [43, 76, 77]. The reliability of DBS is therefore somewhat uncertain. Nonetheless many studies use DBS as the sole source of DNA template for detecting malaria or for testing for resistance genes [67, 78, 79]. As a source of DNA, DBS have the potential to be used to investigate malaria prevalence as well as monitor antimalarial resistance genes and perform genotyping of parasites to monitor whether infections are new or recrudescent [24, 49, 80]. The practical advantages of DBS are the main reason they are an attractive method. Patients or study subjects generally perceive collecting blood drops by finger-prick as less invasive, less painful and a more agreeable procedure than obtaining blood samples by venipuncture [81]. Storage and transportation of DBS is much simpler than for whole blood samples and therefore is attractive for use in remote, rural areas in a resource-poor setting where a cold chain may be lacking.

1.3 Diagnostics and treatment decisions

A clinician's basis for selecting treatment is complex. A febrile child is a diagnostic challenge both because of the challenges with the diagnostic methods and overlapping clinical presentations of illnesses, as outlined above, and other factors such as a child's more limited ability to communicate their subjective experience of their illness. When laboratory facilities are lacking or unreliable, treatment decisions may often target a broad spectrum of etiologies in order to avoid undertreatment and increase survival. The result may be overtreatment with both antimalarials and antibiotics, which drives antimicrobial resistance, especially when suboptimal doses of medication are used or antimicrobial treatment courses are not completed [82-84]. Even when laboratory facilities are present, clinicians may not have faith in the diagnostics done and may treat patients for malaria or other infections despite negative laboratory results [21, 23, 85-87]. The widespread availability of antimalarials over-the-counter in many malaria-endemic countries may also contribute to overuse of the drugs [88-90].

The recommended antimalarial treatment of uncomplicated *P. falciparum* infection is artemisinin combination therapy (ACT) [24]. Resistance has developed in *P. falciparum* parasites to other previous commonly used treatments such as sulfadoxine-pyrimethamine (SP) and chloroquine [91]. SP is still used in intermittent preventative therapy in pregnant women [92], but chloroquine has lost its place entirely in official *P. falciparum* treatment and prevention recommendations [24, 91]. Despite the solid documentation of resistance to these antimalarials they are still available in many pharmacies in sub-Saharan Africa [93]. A recent study by Selemani *et al* showed that only 58% of malaria cases received the recommended treatment with ACT [94]. Febrile illness may in some cases be a medical emergency with

danger signs. In these cases presumptive treatment of various possible causes of illness may be necessary and correct practice.

1.4 Malaria immunity

Children in areas with high malaria transmission have a marked reduced risk of severe malarial disease after their second year of life and may develop some immunity to severe malarial infection already after one or two infections [95]. In areas with low to moderate transmission it may take up to the child's fifth birthday before a significant reduced risk of severe infection is observed [96]. It appears that if exposure to malaria is too low, with too few infectious mosquito bites per year, the immunity an individual has developed will wane [97]. Older children and adults in malaria-endemic areas are less susceptible to developing clinical malaria infection and are able to clear parasites more quickly while receiving antimalarial treatment as well as without antimalarials [98]. Full immunity to malaria has not yet been found and the mechanisms of malarial immunity are complex [99].

1.5 Asymptomatic malaria infection

Asymptomatic malaria infection can pose a challenge in the diagnosis of febrile illness.

It is generally accepted that inhabitants of malaria-endemic countries can have asymptomatic malaria parasitemia [100, 101]. Varying prevalence of asymptomatic malaria parasitemia has been identified in different populations studied with low levels such as 1.7-8.7% shown in one area in Gabon [100], while higher levels of 12%, 27%, and 52% were found by microscopy, RDT, and PCR respectively, among another adult Gabonese population studied [102]. In yet another study, in Gambia, it was concluded that true asymptomatic malaria parasitemia was uncommon in children [103]. In an area in Kenya 33.8-73.8% of a studied population had asymptomatic infection [101] and 33.1% in a study in Nigeria [104]. These variations in prevalence underscore the importance of further studies being performed in

various populations to determine the significance of asymptomatic malaria in different areas. Individuals with asymptomatic malaria parasitemia presenting with febrile bacterial or viral infection may at the same time have a positive malaria test, which can cause clinicians to overlook the true cause of illness. However, there is also evidence that malaria may predispose for invasive bacterial disease [105].

Asymptomatic malaria is not only present in high transmission areas but has also been identified in low transmission settings [106]. A recent study demonstrated that the proportion of subpatent infections detectable by PCR was high in northwestern Tanzania indicating the value of using PCR rather than only RDT and microscopy in research on prevalence of malaria parasitemia in a population [71]. It is especially important to detect asymptomatic malaria infections in an elimination setting [107] as individuals with asymptomatic gametocytemia can act as a reservoir for perpetuating the malaria cycle by infecting mosquitoes. An estimated 14-40% of mosquito infections may come from individuals carrying submicroscopic quantities of malaria parasitemia in an area with a low blood slide malaria positive prevalence of about 3%, according to a study by Okell *et al* [108]. Asymptomatic individuals do not seek medical treatment and therefore can potentially infect mosquitoes over a long period of time. Artemisinin combination therapy has been shown to reduce gametocyte persistence more than some other antimalarials and is therefore a useful drug with malaria elimination in mind [109].

Gametocytemia appears to be higher among asymptomatic than symptomatic individuals [110], which may be because gametocytemia peaks about 7-10 days after the peak of asexual parasitemia with *P. falciparum* [8] and asymptomatic infections are most often longer in duration than symptomatic infections. Gametocytemia is dependent on age and mostly found

in children in areas with high malaria transmission. In areas with lower malaria transmission intensity the association with age is much less [111].

Patients with asymptomatic malaria parasitemia are less likely to develop an acute malaria attack [112]. Asymptomatic malaria parasitemia therefore appears to reflect a form of partial immunity to malaria.

2. RATIONALE OF THE STUDY

Malaria is a disease that affects a large proportion of the world's population. The diagnostics are challenging with many limitations both in clinical and laboratory diagnostics. Newer methods of diagnostics are emerging including increased use of RDTs clinically and PCR in research and mapping of malaria epidemiology. Comparisons of diagnostics have previously been done, but with varying malaria transmission intensity in different areas and variations in performance of the methods used, further studies are required. Asymptomatic malaria parasitemia can theoretically pose a problem in diagnosing malaria in febrile illness as other causes of fever might be overlooked if malaria parasitemia is present concomitantly with another infection. Asymptomatic malaria parasitemia has been identified in many areas and it is therefore relevant to investigate for it in the current setting in order to assist in the interpretation of malaria results from febrile children. Furthermore, asymptomatic malaria represents a reservoir for transmission of malaria, and thus has implications for efforts to eliminate malaria. With increasing use of DBS in malaria research and few studies done comparing more than just a few DNA extraction methods a larger comparison of methods for DNA extraction from DBS using a standardized sample is warranted. Also only few comparisons of whole blood and filter paper as sources for DNA for malaria investigation have been done despite DBS being used in many studies.

3. AIMS OF THE STUDY

Primary aims:

1. Evaluate and compare malaria diagnostics using routine and research microscopy, DBS and whole blood PCR, and RDTs in a resource-poor malaria-endemic setting.
2. Assess the prevalence of asymptomatic malaria parasitemia in the study population.

Secondary aims:

1. To assess the quality of routine malaria diagnostic tests in the study area. (Paper I)
2. To compare the performance of routine diagnostics with thick blood smear microscopy, thin blood smear research microscopy, RDT, and PCR on both whole blood and DBS. (Papers I, III and IV)
3. To assess the association between clinical signs and laboratory confirmed malaria cases, as defined by the various diagnostic methods. (Paper I)
4. To determine the prevalence of asymptomatic malaria parasitemia in the population and its significance for malaria diagnostics if it is present. (Paper II)
5. To assess the utility of DBS as an alternative to using whole blood as a source of DNA for PCR. (Paper III and IV)
6. To identify a reliable and cost-effective method with a low limit of detection for extraction of DNA from DBS. (Paper III)

4. STUDY POPULATION AND METHODS

4.1 Study area

4.1.1 Dar es Salaam and Tanzania

Tanzania had a population of 44,9 million people with 4,36 million people living in the Dar es Salaam region in 2012 [113]. Malaria is less prevalent in large cities and urban areas than in rural areas in sub-Saharan Africa. A recent study by Pond showed a 0.3% malaria prevalence in Dar es Salaam as compared to 3.9% in surrounding rural areas when using blood microscopy [114]. Approximately 73% of Tanzanians live in areas with high transmission of malaria and Tanzania had approximately 5,48 million suspected and confirmed malaria cases in 2011. *P. falciparum* is the dominating species of malaria in this area [2]. The cost of prevention and treatment of malaria in children under five years of age in Tanzania was 0.62% of the country's gross domestic product (GDP) in 2009 [115] and thus represents a considerable expense for the Tanzanian government which spends a total of 7.3% of the nation's GDP on healthcare [116].

4.1.2 Muhimbili National Hospital

Muhimbili National Hospital (MNH) is a large tertiary referral hospital in Tanzania receiving admissions from all over the country as well as local admissions. MNH is located near the city center of Dar es Salaam. It is also a teaching hospital with close contact with Muhimbili University of Health and Allied Sciences (MUHAS), which is a large teaching institution with schools of medicine, dentistry, nursing, pharmacy and public health. Many complicated patient cases are referred to MNH for further management. There are a total of approximately 1500 beds at MNH. The Pediatric and Child Health Department consists of wards for oncology, malnutrition, diarrhea, pediatric surgery, a burn unit, two general pediatric wards and an acute pediatric care unit. In total there are 217 beds in the department.

4.1.3 Magomeni maternal and child health clinic (MCHC)

Magomeni MCHC is a clinic with facilities for basic diagnostic and health promoting work and is located centrally in Dar es Salaam not far from MNH. Children are brought to the clinic by their parent or guardian for vaccination, weighing and malaria testing or other basic diagnostic measures.

4.2 Study populations

An overview of the populations used in the various papers and how they are related to each other can be found in Figure 1.

Children presenting with fever or a chief complaint of fever that were admitted to the general pediatric wards and acute pediatric care unit at MNH in Dar es Salaam, Tanzania from January to June 2009 were recruited for the study after informed consent (Papers I and IV). When comparing the patients that had consented that were included and excluded from the analysis (Table 1), the only variables significantly ($p < 0.05$) different were mosquito net use, AB use within the previous four weeks and length of hospital stay.

At Magomeni MCHC, 108 children that had not had fever or been treated with antimalarials the past four weeks according to their parent or guardian and were coming for a check-up at the health clinic were recruited after informed oral and written consent (Paper II).

Table 1: Comparison of population characteristics of included and excluded patients (Paper I)

Characteristic or finding (N=included/excluded)	Included % or value (range)	Excluded % or value (range)
Demographics		
Age in months, median (N=300/161)	13.0 (1.0-100.0)	12.0 (1.0-99.5)
Gender – female (N=304/164)	42.8	46.3
Weight in kg, mean (N=272/145)	8.7 (1.5-21.0)	9.0 (1.6-22.0)
History		
Referral from other hospital (N=276/97)	67.8	71.1
Mosquito net used (N=262/137)	96.2	85.4*
Convulsions (N=284/157)	23.6	23.6
Duration of illness in days, mean (N=291/154)	10.4 (1-270)	9.4 (1-180)
Antimalarial previous 4 weeks (N=270/150)	62.6	61.3
Antibiotics previous 4 weeks (N=274/149)	74.8	65.8*
Travel previous 4 weeks (N=257/135)	25.7	20.7
Clinical findings		
Tachypnea (N=284/145)	54.9	54.5
Jaundice (N=297/102)	12.1	7.8
Reduced consciousness (N=299/154)	25.1	29.2
Laboratory test results		
Hemoglobin in g/dl, mean (N=302/157)	8.1 (2.2-13.9)	8.4 (1.7-13.4)
Platelet count in K/ μ l, mean (N=299/152)	335.4 (2.4-1114.0)	330.4 (8.0-1185.0)
Length of hospital stay in days, mean (N=304/162)	7.0 (1.0-71.0)	8.4 (1.0-210.0)*
Outcome – dead (N=304/163)	22.4	21.5

*statistically significant difference between included and excluded cases (p<0.05).

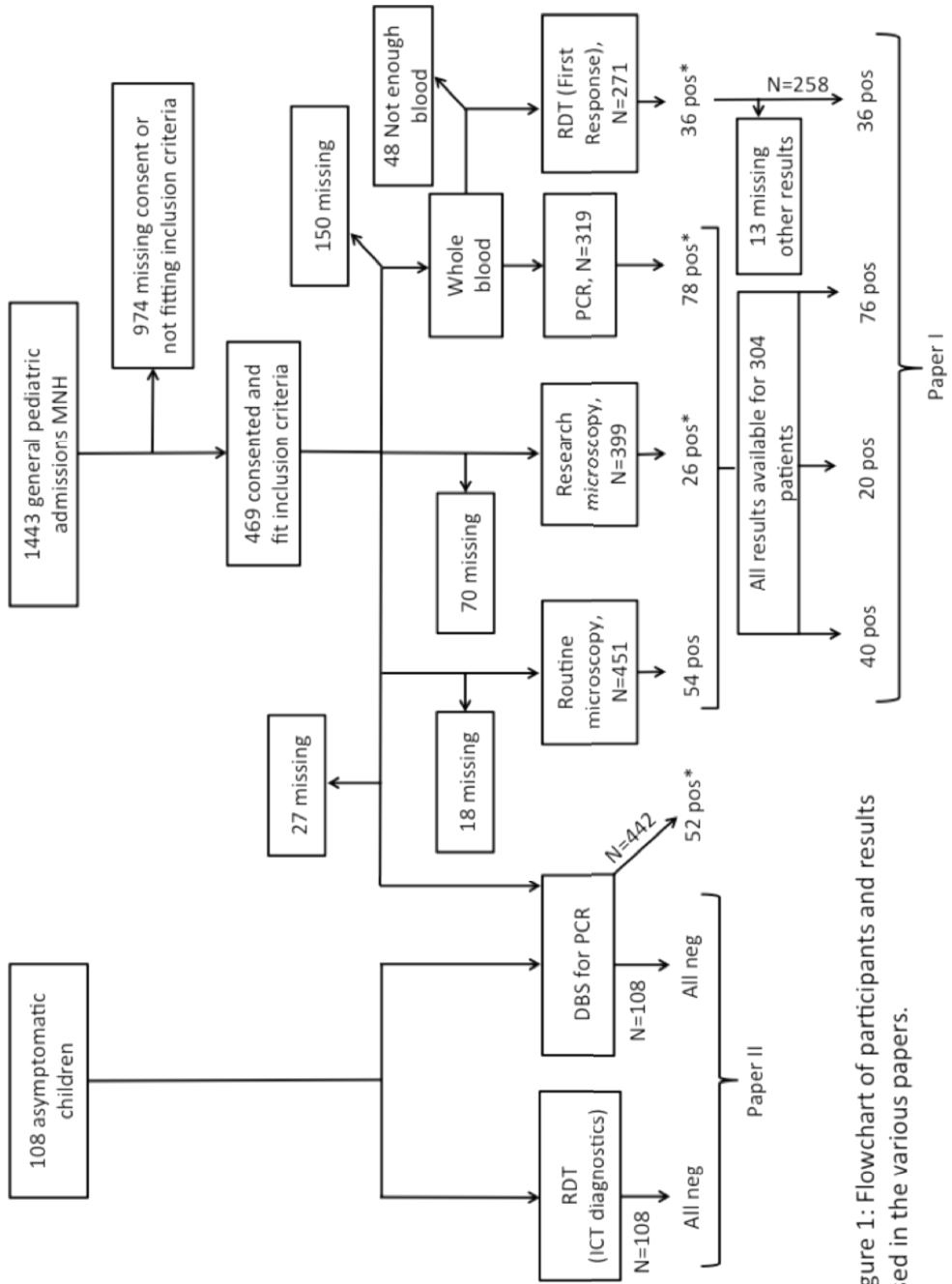


Figure 1: Flowchart of participants and results used in the various papers.

Paper III: 31 of the 442 DBS. *included in Paper IV. MNH, Muhimbili National Hospital; DBS, dried blood spot; RDT, rapid diagnostic test; PCR, polymerase chain reaction; pos, positive; neg, negative

4.3 Study design

The studies for Papers I and II were done as clinically- and laboratory-based prospective, observational studies. The study of DNA extraction methods from DBS (Paper III) and the comparison of PCR on DNA extracted from DBS and whole blood (Paper IV) were methodological studies.

4.4 Material and methods

4.4.1 Social data, laboratory results and collection of materials

Sociodemographic, clinical and laboratory details and results were registered on two different case report forms (CRFs) for the study of febrile children and that of asymptomatic children, respectively (see appendix). The information for the CRF was collected by questioning the parent/guardian and/or consulting the child's medical file. Date of birth, gender, mother's level of education, and whether the child was referred from another hospital, slept under a mosquito net at home, had permanent residence in Dar es Salaam, had known sickle cell disease, had convulsions before admission and how long the child had been ill were recorded. Whether treatment with antimalarials and antibiotics had been given and whether the child had travelled outside Dar es Salaam during the previous four weeks before admission were also documented. Upon admission, weight, axillar temperature, respiratory rate and heart/pulse rate (tachypnea and tachycardia both defined according to pediatric WHO guidelines [117]), spleno- and hepatomegaly, abdominal distension, jaundice, palmar pallor, and level of consciousness were recorded. Hematological data including hemoglobin level, platelet count, erythrocyte count, and leucocyte total and differential counts were recorded along with routine blood slide result, and results of blood culture if done. In-hospital treatment with antimalarials and/or antibiotics, length of hospital admission, as well as outcome dichotomized as dead and alive was documented.

Thick blood smears were stained with Fields stain and examined as part of the hospital routine at MNH. Parasitemia was reported as number of parasites seen per 200 white blood cells. Results of blood slide microscopy for malaria, hematological data as well as clinical observations were recorded.

4.4.2 Research blood smear microscopy

Thin blood smears were prepared at the ward and dried and stored before transport. Retrospectively, the blood smears were fixed with methanol and then stained with 5% Giemsa stain and 100 fields were examined with oil-immersion on high magnification before a slide was deemed negative. Parasitemia on positive slides was reported as percent of erythrocytes infected.

4.4.3 DNA extraction – whole blood

A vial containing EDTA-blood remaining after routine hematological testing was kept frozen at -20°C for 2 to 2.5 years until DNA extraction was performed. DNA was extracted from whole blood using a QIAamp® DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions (Paper I). This method was selected, as it is the routine at the laboratory where the work was carried out and is a reliable method that has performed well in previous studies [44, 118-120].

4.4.4 DNA extraction - DBS

From the febrile children, DBS were collected by dropping two drops of blood from a syringe on a segment of Whatman® Schleicher & Schuell filter paper, grade 589/2 (Whatman GmbH, Dassel, Germany) while collecting routine blood samples (Paper IV). In the asymptomatic malaria parasitemia study, blood collected by finger prick was blotted on a segment of filter

paper. Thereafter the DBS were stored in airtight pockets after completely air-drying. Initially the DBS were stored at room temperature protected from sunlight for three to nine months. Later they were stored at -20°C for approximately 3.5 years until DNA extraction was performed.

The cleaning method of the puncher (Harris Uni-Core™ puncher, Qiagen, Hilden, Germany) between punching circles from DBS was developed based on the manufacturer's and distributor's recommendations and tested to ensure no transfer of parasites between samples (Paper II). Both sodium hypochlorite (NaClO) and 100% ethanol were used in the cleaning process along with punching of blank filter paper. Care was taken to avoid transferring liquid to the DBS during the cleaning process.

Several methods of extraction from DBS were tested and compared using DBS prepared from a 2- and 5- fold dilution series of an external reference sample of *Plasmodium falciparum*, US 04 F Nigeria XII (WHO Specimen Bank at Centers for Disease Control and Prevention in Atlanta, GA USA) in malaria negative blood. For each DBS, 50 μl blood was placed on a segment of the same type of filter paper as for the clinical samples described above. These DBS were air-dried a minimum of overnight before using them for DNA extraction. Seven different methods for DNA extraction from DBS were tested including three different methods using Chelex-100® Molecular Biology Grade Resin (Bio-Rad Laboratories, Hercules, CA, USA) [72, 78, 121-129], two using InstaGene™ Matrix (Bio-Rad Laboratories, Hercules, CA, USA) [74, 127], QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) [68-70, 102, 122] and a method using TE-buffer [72]. The best and most consistent method (detecting the most consecutive dilutions) was used on the clinical DBS that had been stored for approximately four years (Paper IV).

4.4.5 PCR

A genus-specific PCR was done on DNA extracted from whole blood and from DBS using a novel PCR assay targeting *Plasmodium* mitochondrial genome [44]. The same PCR-method was also used for the comparison of methods for DNA extraction from DBS. Species-specific PCR was done on samples positive by whole blood PCR using a method targeting the ribosomal 18S gene [130]. Samples negative by species-specific PCR were sequenced [44]. These PCR methods were chosen, as they are established methods used at the laboratory where the samples were analyzed and had also previously performed well demonstrating high sensitivity and specificity when compared to a reference nested PCR method [44].

4.4.6 RDT

RDTs were performed as instructed by the manufacturer (Papers I and II). Different types of RDTs were used for the two studies. The RDT used in the study of febrile children detected both HRP2 antigen and PAN pLDH antigen despite that only *P. falciparum* was expected. This was useful to assist in classifying cases as current or recent malaria infection and to increase likelihood of detecting all malaria infections. For the study of asymptomatic children an RDT only detecting HRP2 was used. An RDT detecting only HRP2 was considered sufficient because only *P. falciparum* infection was suspected in the population and detecting HRP2 is more sensitive than pLDH and therefore increases the likelihood of detecting asymptomatic cases with low parasitemia. For the asymptomatic study, blood was collected from a prick in the child's finger to perform the RDT. For the febrile children the RDT was performed retrospectively on EDTA-blood that had been kept frozen at -20°C for four years. Both of the RDTs that were used were recommended according to the Global Fund Quality Assurance Policy [131]. The RDT used in Paper II was selected based mainly on accessibility

while the RDT selected for Paper I was based on its performance during WHO RDT testing [51], price and availability.

4.5 Statistical analysis

Statistical analysis was done using IBM SPSS Statistics version 19 (SPSS Inc, IBM company) and statistical significance was defined as p-value <0.05. For categorical variables statistical analysis was performed using Chi-squared test, or Fisher's exact test for analyses of variables with few observations. Multivariate analysis was done using automated and manual backward-stepwise logistic regression on all variables with p-value <0.2 in the univariate analysis. Variables with less than 250 patients with both RDT result and data for the variable being analyzed available, that also were considered less relevant factors based on previous studies and on the univariate analysis, were excluded from the multivariate analysis for predictors of positive RDT (Paper I). Other basic statistics, such as frequency counts, were also performed using SPSS.

4.6 Ethical considerations

A research permit was obtained from the Tanzania Commission for Science and Technology (COSTECH), and ethical clearance was received from the appropriate bodies at MUHAS and MNH and from the Regional Ethical Committee of Western Norway. The study was done in collaboration between MUHAS/MNH and the University of Bergen/Haukeland University Hospital, Norway. The parent or guardian of each child received oral as well as written information, if desired, in English or Swahili. Written consent was obtained from the parent or guardian of each child by signature or fingerprint.

Thin blood smears, frozen EDTA-blood (kept frozen using dry ice) and DBS were transferred, using the MUHAS material transfer agreement, to the University of Bergen in Norway for analysis.

5. SUMMARY OF RESULTS OF PAPERS

5.1 Paper I

Challenges in diagnosing paediatric malaria in Dar es Salaam, Tanzania.

Strom GE, Haanshuus CG, Fataki M, Langeland N, Blomberg B

Malar J 2013, **12**:228.

Objectives: To study clinical and laboratorial diagnostics of malaria in febrile children admitted to the general pediatric wards at Muhimbili National Hospital in Dar es Salaam, Tanzania and also to determine the morbidity and mortality attributable to malaria in the study population. The study aimed to compare various diagnostic methods for malaria.

Results: Among the 304 children included in the study, 62.6% had received antimalarials during the previous four weeks prior to admission, and 65.1% and 96.1% received antimalarials and antibiotics, respectively, during admission. Routine thick blood smears, research blood smears, PCR, and RDTs detected malaria in 13.2%, 6.6%, 25.0% and 13.5% of patients, respectively. Positive routine microscopy was confirmed in only 43%, 45% and 53%, by research microscopy, RDTs and PCR, respectively. Eighteen percent had positive PCR but negative research microscopy. Reported low parasitemia on routine microscopy was associated with negative research blood slide and PCR. RDT-positive cases were associated with signs of severe malaria. Palmar pallor, low hemoglobin and low platelet count were significantly associated with positive PCR, research microscopy and RDT.

Conclusions: The true morbidity and mortality attributable to malaria in the population remains unknown due to the wide discrepancies in results between the diagnostic methods. The current routine microscopy seems to result in overdiagnosis of malaria and, consequently, overuse of antimalarials. RDTs appear to have the potential to improve routine diagnostics. The clinical implication of the many RDT-negative and PCR-positive patients needs to be elucidated.

5.2 Paper II

No asymptomatic malaria parasitaemia found among 108 young children at one health facility in Dar es Salaam, Tanzania.

Strom GE, Tellevik M, Fataki M, Langeland N, Blomberg B

Malar J 2013, **12**:417

Objectives: To investigate whether asymptomatic malaria parasitemia was present in a population parallel to a febrile pediatric population studied at the Muhimbili National Hospital general pediatric wards. Also, to determine the asymptomatic malaria parasitemia prevalence among children in the area in order to improve interpretation of the results of the study of malaria in febrile children.

Results: Of the 108 asymptomatic children included, none had malaria parasitemia detectable by RDT or PCR of DBS. The median age of the children was 4.6 months ranging from 0.5 to 38 months. Nearly all the children were reported to be sleeping under a bed net.

Conclusion: There appears to be no asymptomatic malaria parasitemia in the studied population. However the low median age and the small sample size for the study taken only from one health facility make the results inconclusive and they cannot be generalized to a larger population. Further studies that are larger, with higher age groups and including various locations both rural and urban should be performed.

5.3 Paper III

Comparison of four methods for extracting DNA from dried blood on filter paper for PCR targeting the mitochondrial *Plasmodium* genome

Strom GE, Tellevik MG, Hanevik K, Langeland N, Blomberg B

Manuscript submitted

Objective: Identify the most sensitive and consistent of commonly used methods for extracting DNA from DBS.

Results: The Chelex-100®, InstaGene™ matrix, and QIAamp® kit methods performed similarly resulting in malaria detection in all dilutions to a limit of 0.5 to 2 p/μl while the TE-buffer method performed poorly. The Chelex-100® method using soaking of DBS punches overnight in saponin in phosphate-buffered saline (PBS) proved to be the method resulting in the lowest parasite detection level and most uniform results and was also a cheap method. To compare the two methods with the lowest limit of detection on true clinical samples 31 DBS that were collected from febrile patients four years earlier and were positive for malaria as determined by mitochondrial PCR done on whole blood were tested with both methods. The same clinical DBS (13) were found positive when DNA was extracted from DBS using both the Chelex-100® and InstaGene™ matrix methods with the lowest limits of detection.

Conclusion: Simple and low-cost methods for extracting DNA from DBS can be useful and perform well. They can potentially detect low-grade parasitemia and when combined with a sensitive PCR-method they may prove to be a good alternative to PCR done on DNA extracted from frozen whole blood. Evaluation of the methods with the lowest limit of detection on clinical samples indicates that extracting DNA from clinical DBS may have much lower sensitivity than extracting DNA from whole blood.

5.4 Paper IV

PCR targeting *Plasmodium* mitochondrial genome of DNA extracted from dried blood on filter paper compared to whole blood.

Strom GE, Moyo S, Fataki M, Langeland N, Blomberg B.

Manuscript submitted

Objectives: To compare the results of genus-specific *Plasmodium* PCR on DNA extracted from EDTA-blood and from DBS collected from febrile pediatric admissions four years earlier. The study also aimed to compare PCR of DNA from DBS to thin research blood smear microscopy and RDT results.

Results: Of whole blood samples, 24.5% were positive on mitochondrial *Plasmodium* genus-specific PCR and 11.2% of DBS were positive by the same PCR method. All patients with positive DBS samples were also positive by *P. falciparum* species-specific PCR done on DNA extracted from EDTA-blood. All RDT-positive patients were also positive on PCR of DBS. One patient was positive on PCR of DBS but negative on RDT. Three research microscopy positive cases were negative on PCR of DBS and one of them was also negative on whole blood PCR.

Conclusion: PCR of DNA extracted from whole blood appears to perform superior to PCR of DBS. DBS PCR detected more cases than were detected by RDT and microscopy, though not all cases positive by microscopy were positive by DBS PCR. Because of the many practical advantages of DBS, when it comes to storage and transport, the method will likely remain an alternative that is relevant for research purposes despite the low sensitivity found in this study. Further studies are needed in this area.

6. DISCUSSION

6.1 Discussion of main findings

6.1.1 Routine malaria microscopy

The large misdiagnosis of malaria by routine microscopy of thick blood smears identified in this study (Paper I) has also previously been described in several studies [35, 87, 132]. Only 43-53% of positive routine blood smears for malaria were confirmed positive retrospectively by research diagnostic methods (research thin blood smear microscopy, RDT, or PCR of whole blood), indicating overdiagnosis by routine microscopy. In addition, 55 PCR positive samples were not positive on routine microscopy (Paper I), which indicates underdiagnosis by routine microscopy. Poor quality microscopes and staining solutions along with high workload and insufficient training of personnel have previously been identified as contributing factors to the misdiagnosis of malaria by microscopy and these factors may also have contributed in this setting [34, 133]. Despite the fact that the routine diagnostics performed so poorly, a significant association was found between high parasitemia (>10,000 parasites/ μ l) on routine blood smear and positivity by PCR. This indicates that the cases with high parasitemia, which should not be missed due to their increased likelihood of clinically severe illness [134], indeed were not overlooked by routine microscopy. The many overdiagnosed cases are likely due to microscopists mistaking artifacts or blood components for *Plasmodium* parasites [32], as these were reported as having low parasitemia. The study indicates that “scanty malaria” reported from routine thick blood smear microscopy has a high likelihood of being false positive. There was no significant difference in case fatality rates between cases positive only by PCR and negative by all other diagnostic methods as compared to those positive by other methods. This is likely due to malaria overtreatment in hospital, with approximately two-thirds of all patients receiving antimalarials during admission. In addition, this may indicate that the parasitemia only detected by PCR may be

clinically insignificant remains of parasites post-treatment or asymptomatic parasitemia with concomitant alternative illness. With only 13% of patients being positive by routine blood smear microscopy the high level of treatment with antimalarials indicates that clinicians lack confidence in routine malaria diagnostics. Given the poor accuracy of the diagnostic tests, it is understandable that clinicians do not follow test results, but this also leads to vast overtreatment of malaria. A significantly higher number of PCR positive, routine microscopy negative patients received antimalarial treatment than PCR negative, routine microscopy negative cases ($p < 0.001$) despite the fact that the PCR was performed retrospectively and was not available to the treating physician. The results of this study support the findings of previous studies that routine diagnostics with microscopy are poor in many health institutions and should be improved or alternative methods employed [30, 34-36].

6.1.2 Comparing the study diagnostic methods

Recent studies have identified varying sensitivities of different diagnostic methods for malaria. The same inconsistency between microscopy, RDT and PCR results as was found in Paper I, with PCR detecting malaria in more cases than the other methods, has also previously been described [111, 135-137]. The sensitivity and specificity of microscopy with PCR as the gold standard found in a study by Manjurano *et al* [138] was similar to that reported in Paper I. A limitation of this study is that no true gold standard with definite clinical applicability was identified.

Blood microscopy has long been the gold standard for malaria diagnosis but the results of the current study support the notion, also previously implied [43, 44, 139], that molecular diagnostics have the potential to become the new gold standard and most reliable method for malaria diagnostics. The feasibility of PCR in a clinical setting and especially in low-income

countries is however limited by the method's relatively high cost, labor-intensiveness and that it is time-consuming. Specialized equipment and trained personnel are also required, limiting the applicability of the method. PCR is a molecular technique with several variants including conventional, nested and real-time PCR. Within these given PCR variants the variation in sensitivity and specificity is not only dependent on the PCR method used but also on the primer and target sequence used and the protocol. There are also advantages and disadvantages with each method in how time-consuming and labor-intensive the methods are, risk of contamination, and whether they can provide quantification of parasitemia [140]. Results of studies using different protocols for PCR could potentially vary greatly and to a certain degree be difficult to compare. However, studies done so far comparing PCR methods have shown surprisingly consistent results between methods [141]. Nested PCR is often considered a gold standard among molecular techniques [141]. The PCR method used in the current study was a conventional single round method with a mitochondrial target gene that is present in more copies than many other genes in the *Plasmodium* parasite [44]. This makes the method more sensitive than would be expected for most other single round conventional PCR protocols targeting other genes. It also proved slightly superior when compared to a commonly used gold-standard/reference nested PCR method [44]. This supports the high sensitivity of the mitochondrial PCR when used for malaria detection as was found in Paper I and also assumed when interpreting the results of Papers II-IV.

During microscopy of 100 high-powered fields of a thin blood smear only about 0.006 μl blood is examined and in just as many fields of a thick blood smear, 0.16-0.23 μl blood is examined, which is over 25 times more than by thin blood smear [142]. In the PCR assay used, 2 μl DNA template was used, which is equivalent to 4 μl whole blood. With a lowest parasite detection level of 0.5 p/ μl , theoretically it would be expected that the PCR would

detect as little as two parasites in the added DNA template. When using six 3 mm punches from DBS as the source for DNA template, 2 μ l DNA template is equivalent to approximately 0.7 μ l blood on the filter paper. The whole blood technique therefore analyzes almost six times as much blood as when DBS are used. The PCR methods, whether using whole blood or DBS, analyze more blood than is examined on blood smears. This may account for the higher sensitivity of PCR as compared to microscopy and RDTs. For the RDTs used in the current studies (Papers I and II) approximately 5 μ l blood was used, but RDTs detect antigens rather than parasites and the quantities of blood examined can therefore not be readily compared.

6.1.3 DBS as an alternative to whole blood for PCR

Drying blood on filter paper has become a highly used method in malaria research, however the results presented in Papers III and IV draw the reliability of this method into question. The PCR using DBS as a source of DNA detected a similar number of cases as the RDTs and detected more malaria cases than research microscopy, though three research microscopy positive cases were negative on PCR of DNA from DBS (Paper IV). As collection and transport of whole blood is more difficult, DBS are likely the best source of DNA template for PCR in certain situations despite a lower sensitivity than for whole blood. RDTs have the limitation that not all species can accurately be identified on the spot, however methods have been developed to extract DNA from RDTs for performing PCR and have proven to have similar limits of detection as PCR of DNA extracted from DBS [143]. Further testing can be done on the DNA extracted from the RDT for species-identification in addition to for example genotyping [24], testing for antimalarial resistance [66] or for quantification using real-time PCR. RDTs have also previously been shown to have low sensitivity, often having a limit of detection higher than 200 p/ μ l [51].

The method employing Chelex-100® and soaking in saponin overnight has also previously been proven superior to other methods for DNA extraction from DBS when only a few methods were compared [122] and is frequently used [78, 121, 124-126].

Species identification for the samples was done by using species-specific PCR targeting the 18S gene or sequencing if the species-specific PCR was negative despite positive genus-specific PCR. The species-specific PCR has been shown to be less sensitive than the mitochondrial genus-specific PCR as it detects fewer cases of malaria [44]. We therefore expect that cases negative on species-specific but positive on genus-specific PCR likely have lower parasitemia than those positive by both PCR methods. As all those positive by DBS were also positive on *P. falciparum* species-specific PCR done on whole blood from the same patients, this may suggest that cases with higher parasitemia were consistently detected by DBS (Paper III).

The discrepancy between the DBS and whole blood PCR may have many causes. Previous studies have shown similar results whether whole blood or DBS have been used as sources for DNA template for PCR though few studies on this have been done [43, 76, 144]. One explanation for the discrepancy found may be that the amount of blood used in the template extracted from DBS is almost six times less than in the same amount of template extracted from whole blood (as explained in the section above). Thus fewer parasites are likely to be included in each assay when using DBS as the source of DNA as opposed to whole blood. When considering the results of the testing of DNA extraction methods from DBS it must be taken into account that the preparation of DBS from the dilution series was done in a much cleaner and well-organized environment than the clinical DBS were prepared in. The DBS that were made from the dilutions of the standardized reference blood sample were also used

shortly after preparation (after a minimum of air-drying overnight) for the comparison of DNA extraction methods as opposed to the clinical samples, which were stored for several years before examination. Thus there may be limitations in the applicability of the results of the methodological testing to examination of clinical DBS collected in a research setting with limited facilities. The DBS collected from febrile children for this study were however collected in a very realistic environment for field studies. One study has shown a superiority of DBS that had been stored over 4 years as compared to those stored for a shorter period of time, likely because some PCR inhibiting factors had degraded during storage [127]. It would be expected that when one method for DNA extraction from DBS proved superior to the others, the superiority might remain also after applying the methods to older DBS that all had been stored approximately for the same length of time. In order to further increase the applicability of the results of the study comparing methods for DNA extraction from DBS, the two DNA extraction methods that had the lowest limits of parasite detection were compared on clinical DBS from patients with positive whole blood PCR for malaria before concluding which method truly was superior. This was done to reduce the possibility that a method that proved superior on the standardized samples did not remain superior on the clinical DBS that had been stored for a long period of time and possibly under unfavorable conditions. The two methods that were compared gave the same results. The possibility remains that the limit of parasite detection may not be as low on the stored clinical DBS as for the newly prepared DBS of the dilution series, as the methods for extracting DNA from DBS were not compared on clinical samples with known quantities of parasites and stored for varying lengths of time. The DNA quality may also have been affected by the fact that the DBS had been stored under warmer conditions generally over 25°C for some months initially before they were transported and subsequently frozen at -20°C.

The choice of filter paper may also have influenced the results. The filter paper used in this study has not been verified in other studies and is not specifically produced for this purpose. The results of Paper III with a very low limit of detection of PCR of DNA extracted from DBS prepared on this filter paper supports that the filter paper is suitable for collection of DBS. However, it cannot be determined with certainty whether the filter paper used has a sufficient ability to preserve the DNA in the DBS over time.

The fact that the Chelex-100®/saponin method for extracting DNA from DBS that was relatively inexpensive proved to be superior to the alternative methods (Paper III) supports the sustainability of using DBS for malaria research in low-income countries. Elute cards that were not tested in the comparison done here may prove superior, but due to the higher cost they were not considered relevant for this study.

6.1.4 Implications of findings about diagnostics

RDTs are being used increasingly in routine malaria diagnostics [42]. The findings in Paper I support that the use of RDTs may be a good alternative to microscopy when routine blood microscopy is suboptimal. Though RDTs are not as sensitive as PCR of DNA extracted from whole blood (Paper I), they showed similar detection of malaria cases as PCR of DNA extracted from DBS (Paper IV) and detected more malaria cases than by research microscopy. They also appear to detect the most clinically relevant cases, as positive RDT was associated with clinical signs of severe malaria (Paper I). The limitations of RDTs concerning follow-up of patients and following parasitemia levels during treatment, detecting recrudescence, and discerning certain *Plasmodium* species hinder RDTs in entirely replacing other forms of diagnostics [47-49, 53].

The high number of samples positive on whole blood PCR and negative by all other methods are difficult to explain. PCR has been shown to detect as little as 0.002 p/μl [145], but most other reports have higher limits of detection such as 0.5 p/μl [44] or 1 p/μl [40, 146]. High proportions of submicroscopic quantities of malaria parasitemia identified by PCR have also previously been shown [59, 137]. The high level of submicroscopic malaria reported in Paper I is similar to that found in a study done by Golassa *et al* in Ethiopia [137]. Both studies also identified positivity by microscopy that was half of that found by RDT. These submicroscopic cases may be asymptomatic malaria cases, may represent irrelevant malaria DNA persisting post-treatment in patients presenting with symptoms attributable to another co-infection [147, 148], or the detected malaria parasitemia may be clinically relevant and the cause of the patient's symptoms requiring treatment. The study done on healthy children showed no presence of asymptomatic malaria parasitemia in the population (Paper II). A recent study showed that most malaria infections under effective treatment were PCR negative after approximately 3 days [49], however this study was done using a PCR-assay targeting the 18S gene. Though this may indicate that cases only positive on PCR most likely are not post-treatment cases, the PCR used in this study detecting mitochondrial *Plasmodium* DNA is likely to remain positive for a longer period of time than a PCR assay targeting the 18S gene as gametocytes, which often may persist post-treatment [149, 150], have a higher number of copies of the mitochondrial target gene than of the 18S gene [44]. They may be cases that were pre-treated with suboptimal concentrations of recommended antimalarial drugs or with non-recommended antimalarials causing reduced levels of parasitemia without full parasite clearance. In Paper I it is reported that 63% of the children had been pre-treated with antimalarials before admission and 16% of these had received other antimalarials than what was recommended by WHO. There was no increased case fatality rate among PCR-positive, routine microscopy negative cases that did not receive antimalarials as compared to those that

received antimalarial treatment. More information concerning how long the antimalarials had been used prior to admission, when the treatment had been given and at which doses would have assisted in interpreting these findings. However, in many cases this information most likely would not have been available due to poor memory recall or lack of documentation. A report by Hodel *et al* done in Tanzania indicated that antimalarials were present in the bloodstream of many more patients than those that reported recent antimalarial use [90]. Measuring the concentration of the most commonly used antimalarials in the blood of the children included upon admission would have given additional useful information. Similarly poor memory recall and a desire to please the investigator may have influenced other data collected from the parent or guardian of the participating child.

No significant association was found between positive result on any of the study diagnostic methods (PCR of whole blood, RDT and research microscopy) and case fatality rate (Paper I). This suggests that there are other serious illnesses in addition to malaria in the population accounting for a large proportion of the deaths among study participants. The high total case fatality rate of 22.7% found among febrile patients included in this study was similar to that found in a study on systemic pediatric illness by Blomberg *et al* in 2001-2002, conducted at the same hospital, where a total of 17% of the included patients died and 20.2% of patients with malaria parasitemia died [151]. The high case fatality rate also for children with non-malarial illness despite 96% of patients receiving antibiotics during admission suggests that the many children (two-thirds of study participants) referred from other hospitals may have sought treatment or been referred too late to the tertiary hospital where the study was conducted, treated with inappropriate antibiotics or suffered from other illnesses that were not treated appropriately. The lacking information of other causes of fever than malaria in the study participants is a weakness of this study that was not addressed, as diagnostic methods

for other causes of fever were not consistently available. Information concerning comorbidities could also have been an asset in this study.

6.1.5 Clinical signs of malaria as defined by the various study methods

Thrombocytopenia and anemia were associated with malaria as defined by PCR, RDT and research microscopy. Severity of palmar pallor was correlated to the degree of anemia. Assessing palmar pallor is therefore a useful clinical tool in a resource-poor setting where laboratory facilities may be lacking and hemoglobin levels cannot be measured. This association has also previously been shown for mild anemia when assessed by experienced and trained clinicians [152] and was confirmed by the findings in Paper I. The results reported in Paper I concerning the value of assessing palmar pallor to determine the degree of anemia in children were more promising than previously reported in other studies [153, 154]. It has previously been observed that palmar pallor is superior to conjunctival pallor in assessing anemia in African patients [152].

Various clinical signs were associated with malaria but none of these were specific or sensitive for identifying malaria cases. The findings support that malaria does not present with a clear clinical syndrome that can be diagnosed without laboratory or other testing. Not all clinical or laboratorial parameters were recorded for all patients (Table 1). There may be some concerns about the validity of the clinical data that was collected as the data was collected by various health professionals without any standardization beyond that is was done according to the established hospital routine.

6.1.6 Implications of not finding asymptomatic malaria in the population

Asymptomatic malaria studies that have been done are difficult to compare. Some studies use DBS to identify parasitemia [102, 155, 156], another used whole blood PCR [136], some use RDTs [136, 155], while many studies only use blood smear microscopy [100, 101, 103]. As has been shown in Papers I, III and IV, the sensitivities of these methods vary greatly.

Asymptomatic malaria cases often have low parasitemia though this varies from less than 100 p/µl to over 1000 p/µl depending on the level of transmission in the area and the person's age [157, 158]. Sensitive molecular methods with low limits of detection such as PCR, rather than RDT and microscopy, are therefore likely best to identify these infections as also confirmed by a study by Ganguly *et al* [136]. The finding of no asymptomatic parasitemia in this study (Paper II) is difficult to interpret. It can be considered a confirmation of the strategy presented by WHO to test all patients with suspected malaria and only treat those with a positive test for malaria [24], but it also contradicts the finding in other studies done on asymptomatic malaria which have generally identified asymptomatic cases [100, 101, 103, 159]. A study by Coleman *et al* showed that the RDT they tested was inadequate for identifying low level parasitemia and thus asymptomatic parasitemia [160]. PCR of whole blood would likely have been more sensitive and could possibly have detected asymptomatic cases not found in this study due to the use of less sensitive methods (Paper IV). In addition the small sample size from only one health facility in an urban area with lower malaria transmission than surrounding rural areas may explain the findings.

The use of insecticide treated nets (ITNs) along with increased use of artemisinin combination therapy (ACT) has likely contributed to reducing the burden of malaria in various countries including Zanzibar and northeastern Tanzania [161, 162]. The fact that most of the children included in the asymptomatic malaria study (Paper II) were reported as sleeping under a bed

net at home may contribute to the fact that no asymptomatic malaria was found among the children. However, the question about bed nets in the survey did not specify whether the nets were in good condition, were treated with insecticide or used every night. In addition, some parents/guardians may have over-reported bed-net use in order to please the investigator as coverage according to this study's findings is much higher (97%) than that found in the Dar es Salaam area in a study in 2008 (68%) [163].

The participants in the study of asymptomatic individuals had a very low median age (4.6 months) and were therefore less likely to have acquired asymptomatic malaria than those in the study of febrile children, which had a higher median age of 13.0 months (Paper I) [96, 164]. Younger children are less likely to have submicroscopic quantities of malaria parasitemia as lower parasitemia during malaria infection is linked to increased age [108]. This may contribute to explaining why the level of submicroscopic malaria was so high in the study on febrile patients (Paper I) as opposed to the study on asymptomatic children (Paper II). A weakness of the study design for the asymptomatic malaria study was that the children were only examined at a certain point in time and if any parasitemia had been identified it would not have been possible, without follow-up of the children, to identify whether the children were in a pre-symptomatic phase of infection or truly had an asymptomatic infection [103, 165].

Despite the limitations of both methods used when investigating for asymptomatic malaria, the results of the study may be applied to a population demographically similar to the patient study population to conclude that a positive DBS or RDT result using the same methods as described here most likely is a true clinically relevant malaria.

6.2 General limitations of the study

The study population was small due to time limitations and initial communicational challenges in recruiting patients. In addition, as a consequence of missing information, results, and samples, the sample size was further limited. The study on asymptomatic malaria was meant as a small study to assess the situation and evaluate the methods. In order to obtain more useful and representative results the study population should have been expanded and included a broader age range and participants from both rural and urban areas.

The study setting was an urban area with lower malaria transmission than surrounding rural areas. This limits the relevance of the study of asymptomatic malaria, as asymptomatic malaria parasitemia is less prevalent in areas of low than of high transmission. It also limits the general applicability of the results of the study of febrile children to urban populations similar to the one studied. The fact that the sample collection was done among admissions to a tertiary hospital is also a limiting factor as many uncomplicated as well as severe cases of malaria were likely to have been treated as outpatients or at peripheral hospitals and therefore not included in this study. The population studied is therefore a specialized population and the true applicability of the results may be limited to a smaller subset of the general population due to this selection bias. There are most likely few places in Tanzania that have similar populations to the one studied here. However, the results can be applied to populations admitted to tertiary hospitals in other major cities in Sub-Saharan Africa with comparable levels of malaria transmission.

The time delay between data collection in 2009 and the publication of the first paper in 2013 is another weakness of this study (especially Paper I). Malaria epidemiology is changing and should therefore be monitored frequently and results published promptly to increase their

usefulness. In addition, performing all the investigations without delay after collection, including RDT and PCR of whole blood and DBS, would have improved the results, as storage of biological material can reduce its quality depending on storage conditions and handling during transport. Performing the RDTs upon admission at the hospital rather than retrospectively may have contributed to improving the management of the patients. The RDTs were performed retrospectively due to considerations of funding which were not in place at the time of data collection. The use of the same RDT in both Papers I and II would have increased the comparability of these results.

The choice of PCR method may be questioned, as it is a newer method that has not yet generally been accepted as a reference method and has been used in a limited number of studies. It has, however, proven to be a sensitive and specific method that was a suitable comparative method for this study in which we wished to also identify cases with low parasitemia. Other PCR assays targeting the mitochondrial genome have been used in various recent studies [65, 166, 167].

Including additional methods of DNA extraction from filter paper and performing several parallel extractions using each method on each series of dilutions in the methodological comparison (Paper III) would have increased the validity of the results. The greatest strength of the comparison in Paper III as compared to previous comparisons is the use of a standardized reference sample for the series of dilutions.

The microscopy done retrospectively was done only on thin blood smears because they were available. Examining thick blood smears as well would most likely have increased the

sensitivity of the research microscopy and also increased the comparability to the routine thick blood smear results.

7. CONCLUSION

Malaria remains a disease causing significant morbidity and mortality among children in Dar es Salaam, Tanzania. Diagnostics are challenging but the current study suggests that RDTs may be the best diagnostic method in clinical settings where microscopy is of low quality. PCR is a very sensitive and specific method for research purposes. Using DBS as a source of DNA for PCR circumvents some of the logistic challenges with whole blood PCR and is particularly useful for specimen collection in remote areas, though the sensitivity of DBS is lower than for whole blood. A simple Chelex-100® -based method for extraction of DNA from DBS appears to be a better alternative to the more expensive InstaGene™ Matrix and QIAamp® kit that previously have been considered better alternatives to Chelex-100®. Asymptomatic malaria in young children does not appear to be prevalent in the study area and should therefore not be considered an explanatory factor for positive malaria laboratory results where malaria is less suspected as cause of illness, however the generalizability of these results are very limited due to the many previously outlined limitations of the study on asymptomatic malaria.

8. FURTHER RECOMMENDATIONS

1. Studies using quantitative methods should be done to identify the significance of the submicroscopic malaria cases which were mitochondrial *Plasmodium* PCR positive but microscopy and/or RDT negative. In addition, using a reverse transcriptase PCR for detecting gametocytes may identify whether submicroscopic malaria cases truly are caused by gametocytes as we hypothesize based on the findings in this study.
2. Future studies comparing malaria diagnostics should include LAMP and ELISA (Enzyme-linked immunosorbent assay), as these are methods with increasing relevance. LAMP is especially relevant as an alternative to PCR for molecular diagnostics in studies in resource-poor settings.
3. Larger studies of asymptomatic malaria examining various age groups, as well as comparing surrounding rural areas and the urban area studied in this study, should be performed to determine whether asymptomatic malaria truly is not prevalent in the study area. In addition, whole blood rather than DBS may be a better alternative as a source of DNA from PCR in order to identify the children with very low parasitemia that may not have been found using PCR of DNA from DBS. More specific questions about bed net use, type and quality of bed net as well as specific information about recent antimalarial and antibiotic use should be included in future asymptomatic malaria studies.
4. Further studies comparing methods for DNA extraction from DBS should be done and include various types of filter paper stored under varying conditions for different durations.

5. Larger studies should be done to compare the sensitivity and limit of parasite detection of PCR on DNA extracted from DBS as compared to PCR of DNA extracted from whole blood as only few studies have been done on this and previous studies have showed more consistent results between the two methods than what was found in this study.

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Errata*Paper I:*

Page 3, Results paragraph 2, lines 5 and 6:

The conclusion of no significant association between fatal outcome and PCR, research microscopy and RDT results is correct. However, the case fatality rates should be: 20.0% for PCR, 15.0% for research microscopy and 16.7% for RDT and not the same numbers as given in Additional file 1 as stated.

Additional File 2:

Row two (study microscopy) under the "RDT as gold standard" heading:

The correct numbers are Sens: 55.6%, Spec: 100.0%, False pos: 0.0%, False neg: 44.4%.

Row 4 (RDT) under the "study microscopy as gold standard" heading:

The correct number are Spec: 93.3%, False pos: 6.7%.