Effectiveness of mass drug administration for reducing seasonal malaria transmission towards its elimination in hotspot areas in Zanzibar

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ABSTRACT

**Background:** Following the increase of international funding for implementation of the combined effective malaria control strategies for prevention, diagnosis and treatment in the past decades, a significant reduction with 30% of malaria attributed morbidity and mortality has been achieved. At present, declining transmission intensities, in areas of successful malaria control, have resulted in a relative increase of asymptomatic low parasite density infections that fall below the detection level of rapid diagnostic tests and microscopy. Mass drug administration (MDA) is a treatment of the entire population in a geographic area with antimalarial drugs irrespective of the presence of symptoms and without diagnostic testing, with the purpose of targeting asymptomatic malarial infections.

**Aim:** The overall purpose of this study is to determine the effectiveness of two rounds of MDA for reducing malaria transmission on Unguja island in Zanzibar.

**Methods:** A cluster-randomised controlled study with two arms was performed: an arm with two rounds of MDA, and a control arm without MDA. A total of 7941 field samples from the first MDA round and 9853 samples four months after the second round of MDA were screened. Two different sets of qPCRs, 18S-qPCR and cyt-b-qPCR were used to detect the positivity of *Plasmodium* infection. The species were further identified using restriction fragment length polymorphism (RFLP).

**Results:** Cyb-t-qPCR detected more positive samples compared to 18S-qPCR. The qPCR determined malaria prevalence six months after the second MDA round was 1.7%. *P. falciparum* was the predominant species accounting for 64.6% and 63.3% from first MDA round and four month after second round of MDA respectively. From the first MDA round, the prevalence of *P. malariae* and *P. vivax* was 15.4% with 0.6% respectively. The parasite prevalence in the second round of MDA was: 10.8% in *P. malariae*, 7.2% in *P. ovale* and 0.6% in *P. vivax*.

**Conclusion:** There is still a presence of asymptomatic low-density parasite carriers and the parasite prevalence remains relatively low in Zanzibar. MDA can be effective in low endemic settings when the goal is elimination.
POPULAR SCIENTIFIC SUMMARY

Elimination of asymptomatic malaria infection in Zanzibar

Malaria is a parasitic disease that is caused by a protozoa parasite called *Plasmodium*. There are more than 100 species of *Plasmodium*, five of them (*P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*) can cause malaria in humans and among them *P. falciparum* is responsible for the most cases, followed by *P. vivax*. The disease is life threatening and caused by an infected female mosquito called anopheles that carries the parasite and spread it to humans through bites of the female mosquitoes. Around 216 million people are affected globally and in sub-Saharan Africa the malaria burden is the heaviest, with 90% of the malaria deaths occur. In endemic countries, a significant proportion of the community is infected with malaria asymptotically. One promising way to eliminate malaria is to give the entire population malaria treatment regardless of any symptom or diagnosis. This is called mass drug administration (MDA), which aims to interrupt malaria transmission by clearing the infectious human reservoir including asymptomatic infections. This study employs MDA to investigate the effectiveness of two rounds of MDA on reducing seasonal malaria transmission in the island of Unguja, Zanzibar.

In this study, 7941 and 9853 samples were collected from first round and four months after the second MDA rounds respectively. Those samples were collected from 16 total regions, 8 of these regions were with MDA and 8 regions with no MDA (no antimalarial drugs were given). DNA was extracted from blood samples and was investigated by using highly sensitive methods such PCR(Polymerase Chain Reaction). *P. falciparum* was the most common species with 64.6% from the first MDA round and 63.3% from the second MDA round.

The presence of asymptomatic individuals carrying malaria parasites is still present at low levels in Zanzibar. There is a need for highly sensitive methods for targeting asymptomatic malaria by preventing transmission of malaria infection.
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List of Abbreviations

ACT               Artemisinin-based combination therapy
Cytb               Cytochrome b
DNA               Deoxyribonucleic acid
DDT               Dichlorodiphenyltrichloroethane
DHAp              Dihydroartesinin-piperaquine
FCM               Flow cytometric
HPR-2             Histidine-rich protein-2
IRS               Indoor residual spraying
ITN               Insecticide treated net
LDH               Lactate dehydrogenase
LAMP              Loop-mediated isothermal amplification
MDA               Mass drug administration
MS                Mass spectrometry
Mt-DNA            Mitochondrial DNA
nPCR              Nested PCR
PBS               Phosphate buffered saline
PCR               Polymerase chain reaction
qPCR              Quantitative PCR
RDT               Rapid diagnostic test
RFLP              Restriction fragment length polymorphism
RNA               Ribonucleic acid
rRNA              Ribosomal RNA
SLD               Single low dose
WHO               World Health Organisation
1. INTRODUCTION

1.1 General introduction to malaria and malaria burden

Malaria is one of the most important parasitic diseases. Despite that global morbidity and mortality have decreased substantially during the last fifteen years still around 2000 people die from malaria every day in Africa. Approximately, 3.3 billion people worldwide were at risk of contracting malaria in 2010. The same year, malaria caused an estimated 216 million cases and 90% of deaths occurred, mostly in sub-Saharan Africa among children under five years of age and pregnant women (White et al., 2013). However, malaria cases have decreased from 244 million to 216 million between 2005 and 2010, and a reduction in the mortality of 26% was recorded between 2000 and 2010 (Autino et al., 2012). This parasitic infection occurs in tropical and subtropical areas and is commonly associated with poverty representing a major burden to economic development. (Sachs et al., 2002).

1.2 The Plasmodium parasite

Malaria is caused by a protozoan called Plasmodium. More than 120 species have been described in mammals and five species of the genus Plasmodium can infect humans: P. falciparum, P. vivax, P. malariae, P. ovale and P. knowlesi. Most malaria deaths are due to infection with the genus P. falciparum, which is the most pathogenic and virulent species and the most common species in Sub-Saharan Africa. Also, this genus is associated with drug resistance to various drugs (White et al., 2013).

1.2.1 Life cycle of the Plasmodium

The life cycle of malaria parasites is complex and requires a human host and mosquito vector (Figure 1). The female of Anopheles mosquito transmit malaria to human when taking a blood meal by injecting sporozoites that are present in the mosquito salivary glands. Motile sporozoites, which are injected into the skin of the host, infect the liver cells, where they develop into exoerythrocytic schizonts in the hepatocytes of the liver. The process of invasion of hepatocytes is thought to take 2-3 hours. Inside the hepatocytes, when liver cells rupture, 10 000 – 30 000 daughter merozoites from each schizont are released into blood and invade the erythrocytes (White et al., 2013). Asexual replication occurs in the red blood cells. In the erythrocytes, the parasites go through
several stages and the young rings mature into trophozoites, and finally become schizonts, which rupture the erythrocytes developing merozoites. When an erythrocyte burst, it releases 6-36 merozoites per schizont, which then re-invade new red blood cells and starting a new asexual cycle. Symptoms of malaria are associated with this blood-stage cycle (Mohandas et al., 2012). Some of the merozoites or blood-stage parasites transform into longer-lived sexual forms known as male and female gametocytes. These sexual forms of the gametocytes are ingested by the female mosquito (anopheline) when taking a blood meal. Gametocytes develop into ookinetes that are fertilized forming oocysts in the mosquito mid-gut. These oocysts develop sporozoites that migrate to the salivary glands of the mosquito to invade a new host during a subsequent blood meal (White et al., 2013).

Source: CDC (https://www.cdc.gov/malaria/about/biology/)

Figure 1: Life cycle of malaria parasite

1.3 Clinical presentation and features of malaria

Symptoms of uncomplicated malaria are non-specific and are correlated with fever, making difficult to distinguish from other bacterial and viral infections. Clinical symptoms of uncomplicated malaria include a vague absence of wellbeing, fatigue, headache, muscle aches and abdominal discomfort, which are followed by irregular fever.

Severe malaria is typically caused by *P. falciparum* and is causing acute life threatening form of malaria with high mortality in young children. The manifestations are dependent on age, and occur in immunocompromised patients and small children. Clinical features of severe *P. falciparum* may include severe anaemia and hypoglycemia among children, as well as acute pulmonary oedema, acute kidney injury and jaundice in adults.
Also, all age of groups may get coma (cerebral malaria) and acidosis due to severe *falciparum* malaria. (White *et al*., 2013).

1.4  **The mosquito vector**

The transmission of malaria occurs through the bites of the female *Anopheles* mosquitoes from an infected individual when it takes a blood meal as a prelude to the reproductive process (White *et al*., 2013; Sachs *et al*., 2002). There are more than 500 *Anopheles* species that are recognized worldwide, whereas 70 species are able to transmit *Plasmodium* to human hosts. 41 species are defined as the most important malarial vector worldwide, providing the majority of human malaria cases. Many environmental factors play an important role in vector distribution and malaria biodiversity, i.e. climate seasonality, rainfall patterns, temperature, humidity, presence of vegetation and surface water. Furthermore, human activities, for example agriculture, irrigation, deforestation, urbanization, population movements and wars are linked to transmission levels and malaria epidemiology (White *et al*., 2013).

1.5  **Malaria Diagnosis**

Early and accurate malaria diagnosis is critical for rapid and effective disease management. High-quality malaria diagnosis is essential, since misdiagnosis and delays in diagnosis and treatment can lead to significant morbidity and mortality. Malaria diagnosis involves microscopy or malaria rapid diagnostic test (RDT). The World Health Organization (WHO) recommends promoting malaria diagnosis by using these techniques to identify malaria parasites or antigens in patient blood. Malaria microscopy can estimate the parasite density as well as differentiate the malaria species whereas RDT is a rapid and easy test useful for diagnosis at all levels of health care. RDTs can differentiate *P. falciparum* from the other malaria species.

1.5.1  **Clinical diagnosis**

Clinical diagnosis of malaria is based on the patient’s symptoms and signs, and on physical findings at examination. The first symptom of this parasitic disease is often fever, but there are as well other nonspecific and variable symptoms. The clinical diagnosis of
malaria is challenging, since it overlaps considerably with other common such as bacterial or viral infections. According to WHO recommendations, malaria treatment should always be based on parasitological confirmation by either RDT or microscopy.

1.5.2 Microscopy

Microscopic diagnosis of malaria by using thick or thin blood smears from patients is the “gold standard” for laboratory confirmation and detection of malaria. It has the advantage of differentiating and identify each species of *Plasmodium*. Moreover, this method allows for determination of parasites stages such as gametocytes and the quantification of parasite density.

The classical stain used for malaria microscopy is Giemsa, which gives the parasites a distinctive appearance. The wide usage and acceptance of the microscopy for malaria diagnosis by laboratories all over the world can be attributed to its long tradition of being the only available tool. However, the staining and interpretation processes are time-consuming, labour intensive and the results of the examination depend on good techniques and well-trained technicians (Tanfpukdee *et al*., 2009; Kasetsirikul *et al*., 2016).

1.5.3 Rapid Diagnostic Tests (RDTs)

Malaria rapid diagnostic test (RDT) is a simple, accurate, quick and cost-effective technique that is recognized by the WHO, and is based on immunocromatic detection of parasite antigens, such as lactate dehydrogenase (LDH), aldolase and histidine-rich protein-2 (HRP-2) in a small volume of blood (usually 5 μl) using monoclonal antibodies. This method offers a good opportunity to impact malaria control in the immediate future and to extend parasitological confirmation of this infection to peripheral areas where microscopy cannot be guaranteed. RDTs can differentiate between *P. falciparum* and non-*P. falciparum* infection, or some between *P. vivax* and other species (Mubi *et al*., 2013; Tangpukdee *et al*., 2009; Kasetsirikul *et al*., 2016).

1.5.4 Molecular methods

Traditional malaria diagnosis remains problematic due to a large proportion of individuals carrying asymptomatic malaria parasites with low parasite densities, which often are below the detection limit of both RDT and microscopy. That is why there is a need for molecular techniques that display high sensitivity and high specificity, without
subjective variation. The development of nucleic acid amplification methods, such as polymerase chain reaction (PCR), loop-mediated isothermal amplification (LAMP) and other nucleic acid based methods are generated as new strategies for malaria diagnosis.

1.5.4.1 *Polymerase Chain Reaction (PCR)*

PCR is one of the most specific and sensitive methods for detection of malaria parasites and many PCR methods for *Plasmodium* detection have been published. In 1993, Snounou *et al.* established highly sensitive nested PCR methods, targeting the 18S ribosomal(r)RNA gene of the four *Plasmodium* species (Snounou *et al.*, 1993). Also, probe-based real-time PCRs (qPCR) targeting a highly conserved region of the 18S rRNA genes were developed by Rougemont *et al.*, (2004) and Kamau *et al.*, (2011). Moreover, another nested PCR method targeting the Cytochrome b (Cyt b) gene in the mitochondrial DNA (Mt-DNA) was published by Steenkeste *et al.*, 2009.

Most PCR methods target DNA of the *Plasmodium* and distinguish between different *Plasmodium* species, providing an ideal molecular target for malaria parasite genus and species identification (Kamau *et al.*, 2011).

1.5.4.2 *Loop-Mediated Isothermal Amplification (LAMP)*

The isothermal amplification techniques are claimed to be simple and inexpensive molecular malaria-diagnostic assays that target the conserved 18S ribosome RNA gene or mitochondrial genes of *P. falciparum*. The LAMP reaction is performed under isothermal conditions and requires four specific primers, two inner and two outer primers, in order to recognize six distinct regions of the target DNA. The specificity of LAMP is depending on the occurrence of the amplification reaction when all six regions of the target are recognized by the primers. LAMP has a number of advantages over current methods for the molecular diagnosis of disease. This method offers the ability to detect very small quantities of pathogen in a short time and under isothermal condition. LAMP is a reliable, easy, sensitive, quick and at lower cost than PCR and useful for routine screening for malaria parasite in malaria endemic areas (Tangpukdee *et al.*, 2009; Polley *et al* 2010).
1.6 Treatment of *P. falciparum* malaria

Malaria is a treatable disease, but the treatment depends on disease severity and *Plasmodium* species. Antimalarial drugs are given to eliminate the erythrocytic stages of malaria parasites that cause malarial symptoms.

In uncomplicated *P. falciparum* malaria, artemisinin based combination therapy (ACT) is the recommended first-line therapy in nearly all endemic areas. The first-line treatment for radical cure of *P. vivax* is chloroquine plus primaquine (Cui et al., 2015; White et al., 2013).

Severe *falciparum* malaria should be treated as a medical emergency and requires intensive nursing care and careful management. Intravenous or intramuscular artesunate is the treatment of severe and complicated malaria worldwide (White et al., 2013).

Artemisinins are one of the most potent antimalarial drugs that are effective against asexual and sexual parasite stages. They are the fastest drugs against all erythrocytic stages of malaria stages and can kill parasites within minutes with a parasite reduction ratio of approximately 10,000 per erythrocytic cycle. The mechanism of action is believed to be either oxidative damage to parasite membranes or inactivation of parasite proteins by free radicals produced by Artemisinins (Cui et al., 2009).

Chloroquine is a chemotherapeutic treatment that is used for the treatment and prevention of malaria, especially to treat the uncomplicated form of *P. falciparum* infection. It inhibits DNA and RNA biosynthesis and induces the rapid degradation of ribosomes and the dissimilation of ribosomal RNA. Also, the inhibition of protein synthesis is also observed apparently as a secondary effect. Chloroquine accumulates in very high concentrations in the acidic lysosomal food vacuole of the parasite. The food vacuole is the site of haemoglobin degradation in the parasite and Chloroquine is believed to prevent the detoxification of heme. Chloroquine is thought to disrupt this process by binding to haematin (Ridley et al., 1998)

Artesunate, the water-soluble semisynthetic derivative of artemisinin, is an important agent for cerebral malaria as well as all sorts of other complicated malaria because of its high efficacy and low toxicity. Artesunate has the ability to exterminate ring-stage parasites too rapidly resulting in delayed haemolysis (Zuo et al., 2016).
1.6.1 Antimalarial drug resistance

Drug resistance limits the efficacies of many antimalarial drugs and the parasite will survive and/or multiply although the administration of an antimalarial treatment, resulting in delayed or incomplete clearance of parasites from the patient’s blood, as well as possible treatment failure. Not all antimalarial treatment failures are due to drug resistance, it can also be the result of wrong dosing, problems of treatment adherence, poor drug quality and interaction with other drugs. Antimalarial drug resistance has been documented for *P. falciparum, P. vivax* and recently, *P. malariae*.

The emergence and spread of antimalarial resistance can be considered in two parts: firstly, the initial genetic event, which produces the resistant mutant; and secondly, the subsequent selection process where the survival advantage of parasite in the presence of the antimalarial drug leads to preferential transmission of resistant mutants spreading the resistance of the drug. However, in the absence of the drug, resistant mutants will have a survival disadvantage because of the “fitness cost” carried by the resistance mechanism. Once the selective pressure is removed the prevalence of resistance decline. It has been shown that the “cross resistance” occurs when resistance to one drug might select for resistance to another where the mechanisms of resistance are similar (Cui *et al.*, 2015; Kochar *et al.*, 2008).

1.7 Malaria control and elimination

Effective malaria control describes the situation in which malaria is no longer considered to cause deaths or illness but in which it remains to be transmitted. The aim of malaria control is to reduce the burden of the disease to a locally manageable level where it is no longer a public health problem. Malaria elimination describes the situation in which all local transmission of malaria is interrupted and the incidence of infection in a defined geographical area is reduced sufficiently to prevent re-establishment of transmission.

Vector control is considered to be important for the elimination of malaria in all epidemiological situations. The aim is to protect from infecting mosquito bites and decrease transmission of malaria at local community levels. Insecticide-treated bed nets (ITNs) and indoor residual insecticide spraying (IRS) are two widely implemented interventions. They are the most effective interventions against the anthropophilic, indoor-resting anopheles mosquitoes that are responsible for most malaria transmission in Africa. However, there are many of the areas of low and unstable malaria transmission outside
Africa that are likely to be early targets for malaria elimination programmes, since vector mosquitoes have other behavioural characteristics such as day- and outdoor-biting habits. Other potential role of alternative vector-control tools, such as repellent will be evaluated for use in elimination programmes in these areas (Greenwood et al., 2008).

1.7.1 Mass Drug Administration

One of the possible approaches and main strategies to target asymptomatic malaria infection and thus reducing the burden of malaria with a goal of elimination is MDA. Many asymptomatic individuals act as a reservoir for malaria and a source of infection to others in the population. These people have previously been infected repeatedly and develop enough immunity to control the infection at a low level, which does not cause illness. MDA is a strategy or a treatment whereby the entire population in a geographic area receives an antimalarial drug without diagnostic testing and regardless of the presence of symptoms. MDA was advocated by the World Health Organization (WHO) in the 1950s and 60s for malaria eradication as a strategy when indoor residual spraying (IRS) with dichlorodiphenyltrichloroethylene (DDT) had failed to prevent residual transmission. MDA overcomes the issue of missed infections due to insensitive diagnostic tests. WHO does not recommend MDA for malaria yet, due to insufficient evidence to suggest an overall benefit and the risk of drug resistance development. MDA was a component of many malaria elimination programmes during the mid-twentieth century eradication era. Therefore, MDA is done because a significant proportion of the population harbour Plasmodium parasite without being ill and showing malarial symptoms.

Antimalarial drugs have been administered either directly as a full therapeutic course of treatment or indirectly by the fortification of salt. There is a fear that MDAs will facilitate the spread of drug resistance, which might be decreased or increased depending on the effectiveness of the antimalarial treatment. To reduce the risk of drug resistance development, more than one drug shall be used together for MDAs, which has an effect on gametocytes. In order to have an effect on malarial transmission, MDA requires high coverage of the target population, where a high level of community participation is needed.

As a result, it is important to assess the potential for MDA aiming at reducing malaria burden and transmission, as well as to identify gaps in understanding MDA principle
(Poirot et al., 2013; Greenwood et al., 2008; Newby et al., 2015; Cheah et al., 2016). The purpose of performing this strategy is to target asymptomatic malaria infections and thus reducing the burden of malaria with the goal of elimination. Zanzibar is a good model for the assessment of MDA as being a malaria pre-elimination setting with remaining pockets of transmission (hotspots) and a relatively isolated archipelago.

Cross-sectional screening for malaria prevalence by PCR in all households was performed before the first round of MDA. Also, malaria prevalence four months after the second round of MDA was measured. To estimate the effect of the two rounds of MDA the malaria prevalence by PCR was also estimated at the same time points in a corresponding control area where no antimalarial drugs were distributed.

2. AIM

The aim of this study was to determine the effectiveness of two rounds of MDA with dihydroartesinin-piperaquine (DHAp) + single low dose (SLD) of primaquine to understand whether these two rounds of conducted MDA can significantly reduce the seasonal malaria transmission occurring shortly after the main rains in April-June in Unguja- Zanzibar.

3. MATERIALS AND METHODS

3.1 Study design and sample collection

A cluster-randomised controlled study was performed consisting of two arms, an intervention arm with two rounds of MDA, and a control arm without MDA (Figure 2). A total of 16 Shehias (regions) was conducted in this study where each selected Shehia was randomised either to receive two round of MDA, or to receive no MDA, with a total of eight Shehias in each arm. Two rounds of MDA with Dihydroartemisinin-piperaquine (DHAp) (D-ARTEPP, Guilin Pharmaceutical (Shanghai) Co., Ltd., China) and a single low dose (SLD) (0.25mg/kg) primaquine (Primaquine, Remedica Ltd., Cyprus) were conducted with four weeks interval and were given together, at the beginning of the rain season in April and May.
The first dose of the drugs was given under supervision, and the two other doses were taken without supervision at home. MDA effect was measured by comparing cumulative malaria incidence assessed by PCR in the arm with MDA and the arm without MDA (control) Shehias four months after the last round of MDA. Due to logistical and practical reasons the follow up had to be performed in September at four instead of six months after the second round of MDA. High MDA coverage was measured as the proportion of the population who received the treatment, based on whole population enumeration at the time of the MDA intervention. In a subset of the population post-MDA surveys were conducted seven days after the beginning of each MDA round by measuring piperaquine concentration of a finger prick blood samples, as well as by observing the adverse events for a better understanding of MDA coverage and to evaluate the compliance and safety.

**Figure 2**: Study design (as planned) overview. The study was conducted four months after the last MDA round due to technical issue. Shehias = Regions

### 3.2 Pooling strategy
3.2.1 Samples preparation

In this study, finger prick blood samples from household members at the first round of MDA and four month after the second round of MDA were collected in field from Unguja, Zanzibar. Collected blood samples were taken capillary on filter paper and each sample consisted of approximately 30 µl blood. Approximately, 10 000 blood spot samples on Whatman 3 MM (United Kingdom) filter papers were collected and sent to Karolinska Institutet, Stockholm for determination of the malaria prevalence by molecular methods.

3.2.2 Pooled sample preparation and DNA extraction

DNA was extracted using Chelex 100® boiling extraction method from the dried blood spots (Hsiang et al., 2010). Firstly, four samples were pooled by punching two Ø 3.2 mm punches from each sample into each well of a 96 well plate using a puncher machine (DBS Puncher, Perkin Elmer). In between each sample, the head of the puncher was cleaned with two punches of clean filter paper. In each 96 well plate two P. falciparum positive controls (one control with two punches of 10p/µl + 6 punches of negative and one with two punches of 1p/µl + 6 negative) and one all negative control was included. All eight punches from each well were transferred into a 1.5 ml centrifuge tube using a fine tip tweezers (pincetts) which consider a pooled sample. Each pool samples were incubated with 1ml of 0.2% saponin in PBS for 5 min on shaker at room temperature. The tubes were emptied by removing PBS+ saponin with a pipette (1 ml tips) and the tubes were centrifuged for 5 seconds and the remaining PBS+ saponin was emptied from the tubes with a 200 µl pipette tip. After that, 1 ml PBS was added in each tube (without saponin) and was incubated at room temperature on a shaker for 30 min. As above, PBS was emptied and centrifuged and the remaining PBS was removed from the tubes. Then, 120 µl of freshly mixed 10% Chelex was transferred to each sample and the parasite DNA was extracted by incubating the tubes for 10 min in a 95°C heat-block. The tubes were centrifuged for 5 min at 13.000 rpm and finally, 50 µl of DNA elute were transferred into 96 well plates and stored at -20°C until use.
3.2.3 PCR methods

3.2.3.1 18S qPCR for pooled analysis

Screening of pooled samples was performed to measure the positivity of the samples by using 18S qPCR (Kamau et al., 2011). The PCR amplification was carried out in a 20 µl total reaction volume containing 5 µl DNA and 15 µl master mix. The master mix consisted of 0.25 µM of each primer (Fw: GCT CTT TCT TGA TTT CTT GGA TG, Rv: AGC AGG TTA AGA TCT CGT TCG), 0.125 µM of probe (probe: ATG GCC GTT TTT AGT TCG TG), 1×SsoAdvanced Universal Probes Super-mix (CFX connect Real-Time System. Bio-Rad, USA).
3.2.3.2 Cytochrome b qPCR for pooled analysis

SYBR Green real-time PCR (cytb-qPCR) of 2000 pools was used as described previously (Xu et al., 2015) for parasite detection and for screening in order to target *Plasmodium* cytochrome b gene, with a detection limit of 1-2 parasites/μL blood. The PCR reaction was conducted in 384 well plates with primers (Fw: 5´-TGG TAG CAC AAA TCC TTT AGG G-3´ and Rv: 5´-TGG TAA TTG ACA TCC AAT CC-3´), SYBR Green mix (Bio-Rad, Hercules, CA) and 2 µl of template DNA was used in a final reaction volume of 15 µl. Real-time PCR was performed in an CFX 384™ Real Time System (Bio-Rad, USA) as follows: 95°C for 4 min; 40 cycles of 95°C for 15 s, 60°C for 1.5 min; 72°C for 5 min; and melting curve acquisition. Cytb-qPCR products were confirmed in 1.5% agarose gel electrophoresis with GelRed staining (Biotium Inc., USA). Positive and negative controls were included in each run.

3.3 Single sample strategy

3.3.1 Samples preparation

By using DBS puncher, two Ø 3.2 mm punches from each single sample of all positive pools was punched. In each 96 well plate, two *P. falciparum* positive controls (one with 2 punches of 10p/µl and one with 2 punches of 1p/µl) and one negative control were included.

3.3.2 DNA extraction

The extraction of DNA was performed as previously described for the pooled samples.

3.3.3 Cytochrome b qPCR for single sample

The cytb-qPCR in single sample was performed according to the same protocol as in pooling sample described above. All positive samples, regardless whether the positivity was from either Cytb-qPCR or 18S-qPCR, were considered as positive results. However, those samples with unclear results of both methods were repeated in triplicates. The samples were defined as positive if there was detection in at least 1/3 from both PCR methods (Figure 3).
3.3.4 Restriction Fragment Length Polymorphism (RFLP)

RFLP was used on all positive cytb-qPCR products to determine *Plasmodium* species using single enzyme digestion with one or more of the following four enzymes: FspBI (Thermo Fisher, Waltham, MA), AluI (NEB, New England Biolabs, Hitchin, UK), HpyCH4V (NEB) and Csp6I (Thermo Fisher). All positive PCR samples but negative by RFLP were repeated twice (Figure 3).

The RFLP reaction was carried out in a final volume of 20 µl with 5 µl of amplified qPCR products and 5 units of respective enzyme in 1 × reaction buffer, following the manufactures instructions. In each 96 well plate, positive cytb-qPCR products and four positive controls one for each species (*P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*) and at least one negative control were included. After overnight digestion for FspBI (Thermo Fisher, Waltham, MA) and Csp6I (Thermo Fisher) enzymes at 37°C and 1 hour digestion for AluI (NEB, New England Biolabs, Hitchin, UK) and HpyCH4V (NEB) enzymes, RFLP products were confirmed by using 2% agarose gel electrophoresis stained with GelRed (Biotium Inc., USA) as described previously (Xu *et al.*, 2015).

3.4 Ethical considerations

All participants were informed about the purpose and benefits of the study, as well as the potential disadvantages and side-effects. Additionally, participants were asked to sign an informed consent after receiving the information about the project. The head of households and all the family members were informed concerning study purpose and procedures, and only upon individual explicit written consent could healthcare workers conduct finger prick blood sampling.

4. RESULTS

4.1 Study location and population

The study was conducted in Zanzibar; an Indian Ocean archipelago area located off the eastern coastline of Tanzania and contains a number of several islands. Zanzibar consists of two main islands, Unguja and Pemba, with populations of approximately 900,000 and
400,000, respectively (Figure 4). Zanzibar is considered to be a malaria pre-elimination setting, being a good model for the assessment of MDA (Björkman et al., unpublished). Malaria transmission in Zanzibar is low and highly seasonal. The most cases of symptomatic malaria are recorded in Unguja following the main rainy season which occur between April and June. The peak in malaria transmission is more prominent in Unguja than Pemba. This was the reason why this study was conducted in Unguja only.

This project was conducted almost four months after the second round of MDA and is a part of an on-going study.

![Figure 4: Location of Zanzibar within Tanzania](image)

4.2 The prevalence of *Plasmodium*

In this study, a total of 9853 patients were screened and analysed with the both PCR methods four months after the second round of the MDA. The samples from patients were pooled and all positive single samples were analysed after that.

A total of 16 Shehias was included in the study. Each selected Shehia was randomized either to receive two rounds of MDA, or to receive no MDA, for a total of eight Shehias in each arm of the study. The impact of MDA would be assessed by a) comparing cumulative malaria incidence (passive case detection in health facilities) in the MDA and
control Shehias four months after the second round of MDA (primary outcome) and during the following high transmission season, and b) PCR determined community malaria prevalence four months after the second round of MDA (secondary outcome).

For parasite prevalence, two different methods, SYBR Green real-time PCR (Cytb-qPCR) and 18s-qPCR were used to measure the positivity of the samples from Zanzibar four months after the last MDA round (Figure 5).

Out of the total number of samples, 166 were identified as positive with both the methods. 67 samples were positive with 18s-qPCR and 61 samples of these were as well positive with the Cytb-qPCR. There were additional 6 samples that were also identified as positive by 18s-qPCR only but that did not correlated with cytb-qPCR. Cytb-qPCR identified firstly 93 samples out of the total number of samples in the single sample analysis. All the samples that initially identified as positive in the pooling samples but for unknown reason expressed negative in the single samples in cytb-qPCR were repeated in triplicate. Thus, another 68 samples were expressed positive by cytb-qPCR and the total samples that were identified as positive by cytb-qPCR were 161 (Figure 5). Thus, the parasite prevalence was 1.7% (166/9853).

Additionally, the first round of MDA was performed before this part of study was commenced during the fall 2016. A total of 7941 patients were screened and analysed with the same PCR methods for measuring malaria prevalence. Thus, the parasite prevalence was 131/7941 (1.6%) (Data not shown).
Figure 5: Total number of samples determined by 18S-qPCR and cytb-qPCR assays. Y-axis scale indicates the number of positive samples detected.

4.3 *Plasmodium* species identification

PCR-amplified fragments (positive samples in figure 5) were analysed by using RFLP for *Plasmodium* species determinations. FspBI enzyme was used to discriminate between *P. falciparum* and *P. malariae*, with some identical band pattern for *P. vivax* and *P. ovale*. HpyCH4V or Csp6I is used to distinguish between *P. vivax* and *P. ovale*, but HpyCH4V had the ability to discriminate between all five *Plasmodium* species and *P. falciparum* and *P. ovale* are similar. According to figure 6A, *P. falciparum* were more prevalent when the samples were collected four months after the second MDA round and represent 63.3 % (105/166) of the infections. *P. malariae* was present in 10.8 % (18/166), *P. ovale* in 7.2 % (12/166) and *P. vivax* in 0.6 % (1/166) of the infections. *P. falciparum* mixed with *P. malariae* was present in 6 % (10/166) and with *P. ovale* was 1.8 % (3/166) of the infection.

Figure 6B shows the *Plasmodium* species distribution of first round of MDA from RFLP results. Also, largest percentage was detected in *P. falciparum* with 63.3% and least for *P. vivax* with 0.6%. There was no detection of a mixture of *P. falciparum* and *P. ovale* which was present in second MDA round.
Figure 6A: The RFLP assays for species determination by using restriction enzymes. The figure indicates percentage of species distribution among PCR positives during 2nd round.

Figure 6B: The RFLP assays for species determination by using restriction enzymes. The figure indicates percentage of species determination among PCR positives during 1st round.
5. DISCUSSION

Until the year 2003, malaria burden in Zanzibar was high. Due to the treatment failure of chloroquine, the government of Zanzibar decided to change both first- and second-line treatment guidelines for non-severe malaria to ACT (Artemisinin-based combination therapy). Following deployment of efficacious anti-malarial medicines such as ACT in Zanzibar 2003, as well as several effective malaria control approaches such as IRS (Indoor residual spraying) and parasite-based diagnosis for all age groups by microscopy or RDT, malaria-associated morbidity and mortality as well as malaria prevalence were decreased dramatically with 77%. (Bhattarai et al., 2007). Thus, the prevalence of malaria in Zanzibar, as in most parts of sub-Saharan Africa, has been maintained at low level since 2009 and is considered to be in the malaria pre-elimination phase (Morris et al., 2015).

However, targeting asymptomatic low-density parasite carriers are still a great challenge in achieving malaria elimination in Zanzibar, leading to a risk for further transmission. MDA has been suggested to be an effective approach for targeting asymptomatic sub-patent infections and malaria hotspots by giving an entire population of a malaria affected area antimalarial treatment. The purpose of this study was to determine the effectiveness of two rounds of MDA on reducing malaria transmission in Zanzibar, a pre-elimination setting. The aim is to provide a data on the effectiveness of two rounds of MDA and thus provide evidence for future policy decisions on the usefulness of MDA as an additional tool in the on-going malaria elimination efforts in Zanzibar.

To measure the prevalence of *Plasmodium*, two highly sensitive assays were used for a reliable detection of low-density blood-stage *Plasmodium* parasite in malaria pre-elimination settings. Cytb-qPCR is an ultra-sensitive and high-throughput molecular assay that targets the cytochrome B gene of human *Plasmodium* species presented in mitochondrial DNA and requires only a single round of PCR. The performance of cytbpqPCR was compared with 18s-qPCR, which was used to target the 18S ribosomal(r) RNA gene of the *Plasmodium* species. The study started with a first round of MDA, which was initiated in April 2016, just before the anticipated start of the main rain. The long-term impact of MDA was assessed during the following high transmission season after the rain season. According to a previous study, the MDA is best done in the dry season when
malaria transmission is lowest (Cheah et al., 2016). In this study, the impact of MDA would be monitored several months after the second round of MDA in September (primary endpoint). Due to technical issues, it was impossible to determine the population coverage of the MDA intervention at each round of MDA as well as the baseline demographic characteristics of all participants of study. Therefore, it is unknown whether the PCR-determined malaria prevalence data presented in this study was from intervention arm with two rounds of MDA or from control arm without MDA. The expected outcome from this project was to get higher malaria prevalence in the samples after the rainy season in the arm with no MDA.

The Cytb-qPCR method detected almost all positive samples compared to 18s-qPCR (Figure 5). Although cyt-b-qPCR performed well, there are several limitations with this assay. The cyt-b-qPCR requires agarose gel-electrophoresis for parasite confirming PCR positive samples and RFLP for species determination. A single qPCR for both detection and identification of Plasmodium species would be ideal for molecular surveillance of low-density parasitemia. This was not possible with the cyt-b-qPCR as the high AT-content of the amplified PCR products resulted in low-melting temperatures, which could not be fully distinguished from the melting temperatures of primer dimers (Xu et al., 2015). 18s-qPCR is a probe-based highly sensitive genus specific quantitative targeting a highly conserved region of the 18S rRNA gene of the Plasmodium (Kamau et al., 2011; Rougemont et al., 2004). Farrugia et al demonstrated that mitochondrial targets could be more sensitive than ribosomal ones despite the mitochondrial genes have been less frequently studied, as well as mitochondrial DNA might be better conserved than genomic DNA leading to better targeting of Cytb rather than 18S rRNA genes (Farrugia et al., 2011). Despite 18s-qPCR had a less sensitivity, it detected 6 samples that were missed by cyt-b-qPCR (Figure 5). The differences in the PCR sensitivities may be referred to variations in PCR product sizes, target gene copy number as well as primer and probe binding sites. A few differences in the PCR results might be due to low-density parasitaemias that are likely to be close to the detection limits of the PCRs. Also, DNA extraction by Chelex included boiling at a very high temperature (95°C) might cause fragmentation of genomic DNA and thus PCR sensitivity were reduced. PCR primers and probes have an essential role in PCR efficacy and specificity (Xu et al., 2015). Rougemont et al demonstrated in a report that 18s-qPCR had higher analytical sensitivity due to unspecific amplification and/or probe degradation at higher quantification cycle
numbers. Thus, the lower sensitivity of 18s-qPCR reduces the possibility for targeting low-density *Plasmodium* infections in field samples (Rougemont *et al*., 2004; Xu *et al*., 2015).

The present project also showed that *P. falciparum* is still predominant species in both MDA rounds in Zanzibar with a small number of *P. malariae* infections as well as *P. falciparum* is the most frequent species worldwide. The most important focus in this study was control and elimination of asymptomatic *P. falciparum* infections since asymptomatic *P. falciparum* infection plays a significant role as an infection reservoir in sub-Saharan Africa. However, it has been shown an unexpected detection of *P. ovale* in this study with 4% and 7.2%, respectively in both rounds, since most research was focusing on *P. falciparum*. The reported prevalence of *P. ovale* in sub-Saharan Africa rarely exceeds 5%, and it is only observed in most of the endemic areas outside Africa (Calderaro *et al*., 2012). This observation confirms the sensitivity of qPCR assays that detected *P. ovale* which was not detected by other methods such as RDT or microscopy. RFLP was used for species identification of PCR amplified products.

MDA could be one of the best effective strategy that is used to rapidly reduce malaria morbidity and mortality as well as to reduce or interrupt transmission. Also, MDA prevents relapses and resulting malaria transmission. However, it has been shown that MDA increases and decreases factors contributing to the emergence and spread of drug resistance genotypes and so can either increase or decrease the overall risk depending on this balance. Most MDA recipients are healthy, but some have asymptomatic parasitaemia. These healthy people with asymptomatic parasitaemia clear their infections more rapidly than patients who are ill. The reason might be due to low parasite densities, as well as the immunity that controlled the infection in the first place and allowed persistence of parasitaemia without illness enlarges considerably the antimalarial drug effect. MDA could select antimalarial drug resistance when the emergence and subsequent spread of de-novo resistance requires treatment failure, i.e., recrudescence. Slow drug elimination is one of the risk factor when it comes to resistance selection. However, MDA reduces the risk of selecting antimalarial drug resistance of it reduces the incidence of symptomatic malaria in the treated population, which in turn decreases resistance selection opportunities. Also, the probability of selecting resistance might be increased if MDA is considered to be ineffective because of poor coverage, poor adherence,
substantial migration, or surrounding high transmission and is therefore not currently recommended by WHO. Nevertheless, if MDA is effective then the likelihood of selecting drug resistance should be relatively low (White et al., 2017). Using different antimalarial drug combinations such as DHAp and SLD-primaquine for MDA and individual case management reduce the risk of drug resistance emerging (Grueninger et al., 2013; Cotter et al., 2013). To minimize the risks during the MDA active and passive safety monitoring would be conducted during the study, which in turn was performed in this study.

Finally, the aim of the project was to demonstrate the influence of MDA on seasonal malaria transmission, as well as the advantages of MDA as an approach to eliminate malaria infection in Zanzibar. The social impact of this study involved the decrease of malaria transmission and the improvement of public health in Zanzibar. The symptomatic and asymptomatic parasitic infections would be reduced and cleared. Also, all individuals in the community were aware of the importance of malaria elimination targets in Zanzibar.

6. FUTURE PROSPECTIVE AND CONCLUSION

The present study indicates that the asymptomatic low-density parasite carriers still exist in Zanzibar leading to further transmission. MDAs are more effective in low endemic settings when the goal is elimination and give a direct effect when using several rounds of MDA.

In conclusion, this study is still on-going and what will be taken in consideration includes the determination of the population coverage of the MDA intervention at each round of MDA as well as quantification of low-parasite density by using 18s-qPCR. Also, highly sensitive assays for rapid diagnosis to enable immediate antimalarial treatment to reduce or interrupt the transmission of *Plasmodium* parasites are needed for targeting asymptomatic malaria.
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8. REFERENCES


