PREVALENCE OF SUBMICROSCOPIC PLASMODIUM INFECTION AND GLUCOSE 6 PHOSPHATE DEHYDROGENASE (G6PD) DEFICIENCY AMONG FEBRILE PATIENTS IN BONGA TOWN, SOUTHWEST ETHIOPIA

BEKA RAYA: BSc (BSc in MeLT)

A THESIS TO BE SUBMITTED TO SCHOOL OF MEDICAL LABORATORY SCIENCES, FACULTY OF HEALTH SCIENCES, INSTITUTE OF HEALTH, JIMMA UNIVERSITY; FOR PARTIAL FULFILLMENT OF THE REQUIREMENT FOR DEGREE OF MASTERS OF SCIENCE IN MEDICAL PARASITOLOGY

JIMMA, ETHIOPIA

Aug 2, 2021

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JIMMA, ETHIOPIA

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Abstract

Background: *The identification and management of Submicroscopic infection has become a new and increasingly important challenge for malaria control and elimination. Submicroscopic malaria infections with low parasite density serve as a silent reservoir for maintaining residual transmission in the malaria endemic areas. In addition to this, the lack of Glucose 6 Phosphate Dehydrogenase (G6PD) deficiency enzyme level poses risk to G6PDd deficient individuals when treated with primaquine for prevention of relapses due to liver stage. In Ethiopia, information on the magnitude of sub-microscopic malaria infection and G6PDd among febrile patents are scarce. Thus, the current study aimed at determining the prevalence of submicroscopic infections and frequency of G6PD deficiency among febrile individuals in Bonga town, south west Ethiopia.*

Objective: *The objective of the study was to determine the prevalence of submicroscopic malaria infections and G6PD deficiency among febrile individuals visiting health facilities in Bonga town, south west Ethiopia.*

Methods: *Health facility based cross-sectional study was conducted from July-Oct, 2020. Sample size was 384, convenience sampling technique was used and prevalence of submicroscopic Plasmodium infection was determined by quantitative PCR (qPCR) assay. G6PD deficiency prevalence was determined by using careSTART™ G6PD Biosensor. After checking the data for completeness, SPSS software package version 21.0 was used for analysis. Descriptive statistics was employed to summarize the demographic characteristics of the study subjects. Bivariate and multivariate logistic regression were used to determine the main predictors of malaria infection.*

Results: *Three hundred seventy-two study subjects (with auxiliary body temperature ≥37.5℃) comprising 39.7% and 50.2% of males and females, respectively were enrolled in this study.* The *mean age of the study subjects was 30 years. The overall prevalence of Plasmodium infection by qPCR and microscopy was 110 (29.5%) and 26(6.9%), respectively. The submicroscopic Plasmodium infection was 84(22.5%). Moreover, of the 372 study subjects tested for G6PD, 56 (15%) were G6PDd,* and of this, *53(14.2%) was with intermediate and all variables did not show significant association with Plasmodium infection (p > 0.05).*

Conclusion: *Prevalence of submicroscopic malaria infection was high among Bonga area febrile patients. The results of G6PD phenotyping revealed a modest prevalence of G6PD deficiency among febrile patients in the study area. This could jeopardize the malaria control*

and elimination efforts in Ethiopia. Therefore, to detect and treat this infection in the health facility, advanced diagnostic tools are required.
Keywords: *Sub-microscopic Plasmodium infection, G6PD, Malaria elimination, Ethiopia*

List of abbreviations and acronyms

CQ: Chloroquine

DALY: Disability-Adjusted Life Years

DBS: Dry Blood Spot

DNA: Deoxyribonucleic Acid

DIHA: Drug Induced Hemolytic Anemia

G6PDd: Glucose-6-phosphate dehydrogenase deficiency

MIS: Malaria Indicator Survey

NMCP: National Malaria Control Programme

RBCs: Red Blood Cells

RNA: Ribonucleic Acid

SDG: Sustainable Development Goals

SNNPR: Southern Nations, Nationalities and Peoples' Region

SNP: Single Nucleotide Polymorphism

SSA: sub-Saharan Africa

WHO: World Health Organization

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CHAPTER ONE

1. INTRODUCTION

1.1 Background

Malaria is caused by *Plasmodium* parasites. The parasites spread to people through the bite of infectious female *Anopheles* mosquitoes. There are five *Plasmodium* species that cause malaria in humans, *Plasmodium falciparum, P. vivax, P. malariae, P. ovalae and P. knowlesi* (1). Two of these species *P. falciparum* and *P. vivax* pose the greatest burden. (2,3). *Plasmodium falciparum* is mostly found in the warm and moist parts of Africa. It is the most deadly species found throughout Africa with a unique crescent-shaped gametocyte. It accounts for about 50% of all malaria cases and relapse do not occur from infection by this species. However, a recrudescence of the disease may follow up to a year. The merozoite of *P. falciparum* can invade erythrocytes of any age and more red blood cell is infected than other forms of malaria. *Plasmodium vivax* is the dominant malaria parasite in most countries outside of sub-Saharan Africa (SSA) (4). It is widely spread out because it can develop in guts of mosquitoes at a lower temperature, and it is the cause of the most malaria infections. Sometimes referred to as benign malaria, the cycle of paroxysm happens every 3 days, and the patients generally survive even without antimalarial drug (5). About 43% of malaria in the world is caused by *P. vivax* and the merozoites invade only young erythrocytes, the reticulocytes are unable to mature in red blood cells. Merozoites can only penetrate erythrocyte with mediated receptors known as Duffy blood groups characterized by having, two co-dominant alleles FY^a and FY^b (5).

A symptom of malaria is known by acute febrile illness (4). In a non-immune individual, symptoms usually appear 10–15 days after infection. The first symptoms including fever, headache, and chills may be mild and difficult to recognize as malaria. If not treated within 24 hours, *P. falciparum* malaria can advance to severe illness, often leading to death (3,6).

Malaria transmission is periodic in most parts of Ethiopia, with irregular transmission and prevalence patterns affected largely by altitude, rainfall, and population movement. In General, areas located less than 2,000 meters above sea level in altitude are considered malarious areas. The enormous scale-up of malaria control interventions, including case diagnosis and treatment, distribution of insecticide treated nets (ITNs), and indoor residual spraying (IRS) of houses with insecticides have specially targeted these areas in Ethiopia. About 75% of Ethiopia's landmass is endemic for malaria (7, 8). Generally, major malaria transmission follows the main rainfall

season (July - September) each year. However, many parts in the south and west of the country have a rainfall season starting earlier in April and May or have no distinctly defined rain season (9). Due to rainfall patterns and topography transmission tends to be highly hetrogeneous spatially within and between years. In addition to this, malaria transmissions in Ethiopia is known by cyclic epidemic occurring every 5-8 years, the latest one was between 2003 and 2005 (10,11, 12). Malaria control and elimination strategies depend on providing appropriate treatment of parasitological confirmed clinical malaria cases. Such specific treatment depends not only on accurate diagnostics but also on effective drugs (13). Accurate diagnosis is crucial for treating suspected malaria cases (14). Currently, it is not possible to diagnose sub-microscopic infection, yet developing new or alternative tools to do so is important because transmissions caused by submicroscopic infection makes elimination of this parasite more difficult (13). Thus, RDTs and Microscopy alone may not be good enough for detection of submicroscopic infection in elimination targeted settings.

On the other hand, Glucose-6-Phosphate Dehydrogenase (G6PD) is an enzyme that is important in the Pentose Phosphate Pathway (PPP) and essential for basic cellular function. G6PD also aids in producing compounds to prevent build-up of reactive oxygen within red blood cells (15). G6PD deficiency is the most common enzymatic disorder of red blood cells, affecting 400 million people worldwide (16). G6PD deficiency leads to free radical–mediated oxidative damage to red blood cells, which in turn causes hemolysis. It is an X-linked disorder with high prevalence particularly in people of African, Asian, and Mediterranean descent. The gene encoding the G6PD protein is polymorphic, with more than 400 variants. In sub-Saharan Africa (SSA), this variants wild type G6PD* B, G6PD*A and G6PD*A- were most commonly occur (17). A systemic review revealed that G6PD variants have obvious geographic patterns. The G6PD variants were relative homogeneous in America, Africa, and Asia; sharply contrasted with the heterogeneity of variants in China and Southeast Asia. Among the populations in America, Africa, Yemen and Saudi Arabia, G6PD*A- variant was predominant. By contrast, high variant heterogeneity was observed among the populations from East Asia including China and the Asia-Pacific where no single variant predominated (18).

The clinical manifestations of G6PDd vary from asymptomatic individuals to patients with acute hemolytic anemia, drug-induced hemolytic anemia, favism, and neonatal jaundice (19). The spectrum of hemolytic anemia ranging from mild to severe hemolysis in response to oxidative stress. The likelihood of developing hemolysis and its severity depends on the level of the

enzyme deficiency, which in turn depends on the type of G6PD variant. Patients with G6PD-deficient alleles have selective advantage against severe malaria. (20) The prevalence of G6PD deficiency (G6PDd) is high in different geographical areas where prevalence rate of malaria is currently or was previously endemic (21). Primaquine is the recommended drug to eliminate *P. vivax* hypnozoites and to prevent relapses. Apart from *P. vivax* malaria, primaquine also interrupts *P. falciparum* transmission by killing malaria parasite sexual stages (22). The elimination agenda set by malaria endemic African nations,(23), may rely on primaquine both as a hypnozoitocide for *P. vivax* and as a gametocytocidal for *P. falciparum* will enhance zero transmission.

1.2 Statement of the problem

Malaria occurs in many regions of the world. Despite some reduction in the global burden of malaria in recent years, the disease is still a serious public health concern. In 2018, an estimated 228 million cases of malaria occurred worldwide. According to 2019 world malaria report, there were no global gains in reducing new infections over the period 2014 to 2018. And nearly as many people died from malaria in 2018 as the year before. The WHO warned that disruptions to malaria prevention and control caused by the COVID-19 pandemic may result mortality due to malaria double this year(2).

Africa accounted for 93% of malaria cases and 94% of malaria deaths. Today, despite the documented successes in reduced morbidity and mortality due to malaria, it still remains for taking the life of under five child in Africa every two minutes. It is often stated that the continent bears over 90 percent of the global *P. falciparum* burden (25). This parasite has caused a heavy mortality toll on Africa's population (26). In sub-Saharan Africa, it is a leading cause of death, illness, and poor growth and development among young children. The scale-up of malaria control interventions has substantially decreased malaria burden and transmission across many malaria-endemic countries over the past 15 years with the number of malaria infections and related mortality significantly dropped (24). Moreover, the distribution of *P. vivax* is concentrated mainly in the Horn of Africa, covering Djibouti, Eritrea, Ethiopia, Somalia, and Sudan.(3)

Malaria is a serious public health problem in Ethiopia with an average of two million cases a year (27), The disease is endemic in 75% of the country landmass and 60% of the population are at risk of infection with 54 (6.4%) districts having high transmission (8, 27). As malaria prevalence declines to the level now seen in Ethiopia, malaria transmission is likely to be more and more focal, with some areas being relatively free and other areas still having substantial risk of malaria. Ethiopia now faces the challenge of further addressing this increasingly focally distributed disease. In 2019, a total of 904,496 malaria cases (laboratory confirmed) were recorded, responsible for 213 deaths (24, 29). Of these total cases, *P. falciparum* accounted for 81%, and the remaining were due to *P. vivax* (24).

Despite plenty of studies on the clinical malaria disease, submicroscopic malaria infections are still poorly recognized (28). In malaria endemic countries, a large proportion of *Plasmodium*

infections are asymptomatic or sub-clinical (30). Sub-microscopic malaria remains stand against on malaria control programs as it significantly influences transmission dynamics, and has major health impact on sub-microscopic carriers (31, 32). There is evidence that these sub-microscopic infections contribute to disease transmission, with insect direct/indirect feeding studies demonstrating human-to-mosquito transmission in the absence of microscopically detectable parasites (33, 34). Studies documented the submicroscopic *Plasmodium* infections contributed 20%–50% of all human-to-mosquito transmissions when transmission reaches low level (35), and a meta-analysis of community-based study has shown that microscopy only detected 50% of the infections identified by PCR (36). Together, this revealed the importance of submicroscopic *Plasmodium* infections for ongoing malaria transmission and highlighted the relevance of addressing such infections. These infections should be point out and targeted to enhance malaria elimination. The conventional diagnosis tools such as light microscopy and rapid diagnostic kits (RDT) frequently fail to detect submicroscopic infections. Therefore, high sensitive molecular techniques such as PCR should be employed when malaria transmission approaches zero case and it is more appropriate to find out low density infections.

In addition to this, elimination of *P. vivax* malaria is challenging because relapse infections caused by *P. vivax* hypnozoites can initiate transmission at any time (13, 19). Primaquine is the recommended drug to eliminate *P. vivax* hypnozoites and prevent relapses. However, the lack of G6PD enzyme level information poses risk to *P. vivax* patients when treating with primaquine. Hence, individuals who are to use antimalarial drugs should be screened very carefully for G6PDd. For malaria treatment, screening G6PDd is important so that anti-malarial drug can be given safely to G6PDd deficient individuals (37). To date, there is limited information on the prevalence and distribution of G6PD deficiency in foci areas in Ethiopia (17). Few studies indicated the absence of G6PD deficiency in highland areas of the country, while in lowland areas the prevalence ranged from 1.4 to 14.3% in the southwest (Nuer and Anuak) and northeast (Aregoba in Afar) regions concentrated in certain ethnic groups (38, 39). The government of Ethiopia targets to eliminate malaria by 2030 (35). Major challenges for malaria elimination efforts are submicroscopic malaria infections, relapse cases of *P. vivax* and residual transmission (9, 36). Therefore, the aim of this study was to determine the prevalence of sub-microscopic infections and G6PDd among febrile individuals seeking treatment in health facilities at Bonga, Ethiopia.

1.3 Significance of the study

Currently, many submicroscopic malaria infection cases have been reported in both stable and unstable transmission settings. Many lives can be saved by early detection and treatment of individuals has submicroscopic plasmodium infections. Therefore, determination of submicroscopic malaria infection and screening of G6PD deficiency among febrile individuals in low transmission areas is crucial for malaria elimination and also contribute to generate evidence-on the prevalence of submicroscopic infections and G6PD deficiency in Bonga area, Ethiopia.

CHAPTER TWO

2. LITERATURE REVIEW

Malaria is one of the major tropical diseases that adversely affect the health of people and the economic development of many developing countries, particularly in East Africa (40). Malaria accounts for up to 60% of all health facility visits in the eastern African region. However, due to not good enough health care coverage and other factors, much of malaria-related illness and death actually occurs in the home, therefore, going unreported (41). Detailed description of submicroscopic *plasmodium* infection in low transmission areas is crucial for elimination.

A study conducted on a patients at a tertiary care hospital in Jazan, a low transmission district, southwestern Saudi Arabia (42). Malaria infection was identified in thirty patients by the PCR. Of those *plasmodium* infections, 23.3% was sub-microscopic. Malaria infection were detected additional in febrile than in non-febrile patients (P=0.01). This study recommend that incorporation of molecular tool into the classical *plasmodium* infection detection methods is helpful in low transmission seating area (42).

Another cross-sectional study, which was, conducted among febrile patients in low transmission areas in Dielmo and Ndiop Senegalese villages. Of the one hundred fifty blood samples screened for the presence of *plasmodium* parasites, 20.66% were positive by light microscopy whereas qPCR detected in 47.33% of screened samples. The prevalence of submicroscopic infections was 26.66%. The study detected a high submicroscopic among patients within the study areas (43).

Submicroscopic *plasmodium* infection is common in both high and low-endemicity settings, however its clinical consequences unclear. A cohort of 364 children <10 years and 106 adults was followed for six years in Tororo District. Participants who presented with fever was performed by light microscopy and loop-mediated isothermal amplification. The proportion of visits with submicroscopic infections was 25.8% in childeren and 39.2% in adults. After stratifying by age, Submicroscopic malaria infections were associated with fever in Ugandan children. (44).

According to a cross-sectional study, which was conducted at low transmission setting of Punjab India detected submicroscopic malaria infections by PCR, which would have remained undetected by microscopy diagnosis. Positivity rate of microscopy and PCR was 4.4% and

5.75%, respectively. Conventional methods failed to detect mixed infections whereas 0.26% cases were found mixed infection by PCR. Compared to Microscopy PCR has detected 1.3% additional positive cases. Of the total positive cases identified by PCR, 23.4% infections were found to be submicroscopic, which could not be detected by Microscopy diagnosis. This is particularly important because Punjab state is in malaria elimination phase and targeted to achieve elimination in 2021 (45).

The poor microscopy results suggest the need for more sensitive diagnostic tools for detection of sub-microscopic infections. This study underscores the importance of updating the malaria guidelines to detect and treat sub-microscopic malaria infections (46). Conventional methods can miss a substantial proportion of *Plasmodium* infections in surveys of endemic populations, especially in areas with low transmission of infection. The magnitude of the submicroscopic reservoir needs to be considered for effective surveillance and control (47).

In fact, in most health facilities, quality assured microscope and RDT diagnose the majority of symptomatic patients, thus guiding treatment. However, with the continuous reduction of malaria transmission and progress towards elimination, both microscopy and RDT lack adequate sensitivity to detect low-density parasitemia, leading to an under estimation of parasite prevalence and malaria-related morbidity where infections are commonly submicroscopic, asymptomatic or sub patent in many settings (48, 49).

The rapid advances in nucleic acid testing (NAT) commonly used in research settings have led to highly sensitive, specific and quantitative molecular diagnosis for malaria, and have progressively revealed the widespread presence of submicroscopic Plasmodium infections in both febrile and asymptomatic patient's (48, 50). To date, very few studies are available on the prevalence of submicroscopic infections among febrile individuals in Ethiopia.

It is now well-known that some anti-malarials such as primaquine may induce severe hemolysis in people with G6PD deficiency. Antimalarial drug prescriptions must, therefore take into account the patient's G6PD status in malaria endemic areas such as Ethiopia, where the prevalence of this genetic abnormality is relatively low. Although great clinical heterogeneity is observed depending on the molecular nature of the deficiency and the residual enzyme activity in the red blood cell, there is very poor data on the prevalence of G6PD deficiency and the distribution of involved genetic variants in Ethiopia.

A community-based G6PDd survey conducted among 1,734 spatially unique sites across malaria endemic countries showed that the highest median G6PDd allele frequency (peaking at 32.5%) was in Sub Saharan Africa and the Arabian Peninsula; and lowest median G6PDd allele frequency (<1%) in malaria endemic areas of America (51).

A systematic review, conducted in Burkina Faso showed that an average prevalence of 16.6% and 6.5% of G6PD deficiency was found respectively in men and women in this systematic review. G6PDd was significantly higher in men than women and this systematic review suggests that a systematic screening of the G6PD deficiency is also needed to prevent the occurrence of hemolytic accidents (52).

A hospital-based cross sectional study conducted in 449 febrile patients attending Gambella hospital of Ethiopia showed that the prevalence of malaria was 266 (59.2%). G6PDd phenotypes were also screened using Care Start™ G6PDd screening test. Overall, 33 (7.3%) individuals were deficient for G6PD enzyme activity. Although the prevalence of G6PDd was slightly higher among males 18 (8.6%) than females 15 (6.3%), the difference was not statistically significant. However, G6PDd was significantly higher among the native ethnic groups (Anuak (12%) and Nuer (14.3%)) than the 'highlanders'/settlers (39).

Likewise, a cross sectional study conducted in Tigray, Afar, Amhara, Somali, and SNNPR, who participated during MIS of Ethiopia 2011, identified only the G6PD*A molecular variants (G6PD*A376G mutations) in the study site. This finding supports the safe use of those oxidative agents that have therapeutic effects especially antimalarial agents (e.g., Primaquine) at the single low dose for transmission interruption of *P.f* gametocyte and radical cure of P.v, as a part of malaria elimination toolkit in selected malaria endemic areas of Ethiopia.

However, further study should be conducted to make sure that safe use of those oxidative-agents drugs, chemicals and others, which have important values (therapeutic effect) for managing and treating G6PD deficient individuals (52). In the context of malaria elimination, diagnosis of malaria in individuals with very low parasitemia is not feasible with existing diagnostic tools such as RDT and microscopy, but it requires tools with high limit of detection such as molecular assays that target all infections including asymptomatic and submicroscopic infections (53). The main aim of this study was thus to determine the prevalence of submicroscopic infections and G6PDd among febrile individuals in Bonga town, Ethiopia.

CHAPTER THREE

3. OBJECTIVE

3.1. General objective

To determine the prevalence of submicroscopic malaria infection, assess associated risk factors and G6PD deficiency among febrile individuals at Gebretsadiek Shawo General Hospital, Bonga town, Southwest Ethiopia.

3.2. Specific objectives

- To determine the prevalence of submicroscopic malaria infections among febrile individuals
- To assess associated risk factors for *Plasmodium* infection among febrile individuals
- To determine the prevalence of G6PD deficiency phenotype among febrile individuals
- To determine the sensitivity and specificity of microscopic compered to QPCR

CHAPTER FOUR

4. MATERIALS AND METHODS

4.1. Study area and period

The study was conducted in Bonga town, Bonga district, Southwest, Ethiopia located in Keffa Zone of the Southern Nations, Nationalities and Peoples Region. It lies approximately at 7°16′N 36°14′E with an elevation of 1,714 meters above sea level. The climate is sub-humid, with rainfall during most months of the year. There is only a short dry season. In Bonga, the mean annual temperature is 19.4 °C and the mean annual rainfall is 1787mm. Bonga is the administrative capital of Keffa Zone, and is divided into 8 kebeles.

According to the 2007 Census conducted by CSA, the projected total population of the town was estimated to be 39,800 in 2015. The majority of the inhabitants (72.53%) are Orthodox Christians, followed by Muslims (11.17%), Protestants (9.85%), and Catholics (6.18%) (54).

Bonga town has one general hospital and one health center. This study was based in Bonga Gebretsadiek Shawo General Hospital Bonga town. In 2017, the Ethiopia FMOH updated the country's malaria risk strata based on API, calculated from micro-plan data from more than 800 districts, with strata as shown and Bong town was classified as low malaria transmission area.

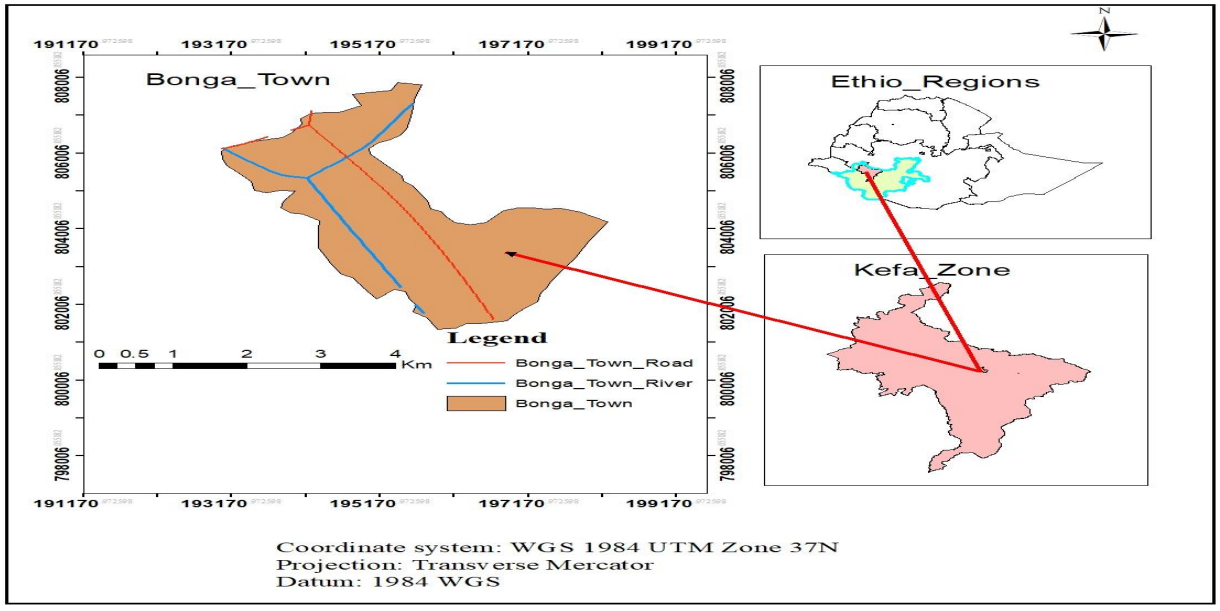


Figure 1: Map of the study area

4.2. Study design

A health facility based cross-sectional study was conducted in Bonga Gebretsadiek Shawo General Hospital from July 01 to Oct 30, 2020

4.3. Population

4.3.1. Source population

All patients visiting Bonga Gebretsadiek Shawo General Hospital laboratory, Bonga Town, Ethiopia

4.3.2. Study population

Selected febrile patients visiting Bonga Gebretsadiek Shawo General Hospital laboratory during the study period in Bonga town, south west Ethiopia

4.4. Inclusion and exclusion criteria

4.4.1. Inclusion criteria

- Presence of fever (Axillary body temperature >37.5^{0}C) at the time of visit

4.4.2. Exclusion criteria

- Individuals taking anti-malarial drugs within the past two weeks

4.5. Study variables

Dependent variable

- Submicroscopic malaria infection
- Prevalence of G6PD deficiency

Independent variables

- Age
- Sex
- Place of residence
- Educational status
- Occupation

- ITN utilization
- IRS
- Pregnancy
- Auxiliary body temperature

4.6. Sample size and sampling technique

4.6.1. Sample size

The sample size was determined based on single population proportion formula as follows:

$$n = \frac{Z^2 P(1-P)}{d^2}$$

Where; n = sample size, Z = Z statistic for a level of confidence (for the level confidence of 95 percent, Z value is = 1.96), and **d** is the margin of error (**d**=5%), P is the sample proportion. There is no previous study in the area the sample size was determined by using P= 50%. The sample size taken with the value of P=50% is to be 383.

$$n = \frac{1.96^2 x 0.5\ (1-0.5)}{0.5^2} = 383$$

4.6.2. Sampling technique

Convenient sampling technique was used to select the study participants during the study period.

4.7. Data collection instruments

4.7.1 Socio-demographic data collection

Data were collected using semi-structured questionnaire. The questionnaire was developed in English and then translated to Amharic (local language of the study areas) and back translated to English. Comparisons was made to ensure consistency and completeness between the two versions. Data were collected from each study subjects by trained data collectors who are conversant of the Amharic language, using a pre-tested, semi-structured questionnaire. The questionnaire was developed to elicit information on the socio-demographic variables, availability and utilization of bed nets, previous malaria history. For children the questionnaire was completed by interviewing their parents or guardians.

4.7.2. Blood sample collection protocol

Blood samples were collected by finger prick using disposable lancet by trained laboratory technologist following standard WHO protocol. Finger prick blood sample approximately 200µl was collected using 300µl EDTA capillary tube from each febrile cases as per WHO guideline (56)

4.7.3 Blood film preparation and Microscopic examination protocol

Thin and thick blood films were prepared for microscopic examination of malaria parasites. Two blood smear slides were prepared for each study subjects according to the standard protocol on site (57). Both thick and thin blood films was made on a single slide. First blood smeared slides were air dried then fixed with absolute methanol and stained at the hospital with 10% Giemsa for detection and quantification of malaria parasites (Annex 5). Then the stained slides were examined microscopically using a 100x oil immersion.

The blood smeared slide was considered "positive" if malaria parasites detected in the smear and considered "negative" when the parasites are not detected after examining 200 oil immersion fields of the thick smear. When malaria parasites are detected in a blood film, the parasite density was quantified by counting the number of parasites against 200 white blood cells in a thick smear assuming an average total WBC count of 8000/µl of blood (58, 59) (Annex 6). The thin smears were examined to identify the *Plasmodium* species. The type of parasite species identified and the parasite density was recorded. All blood films were initially read on site or at the hospital laboratories by trained laboratory technologist. Films positive for parasites and 10% of the negative slides was subsequently read by blinded lab technologist at JU-TIDRC research laboratory.

4.7.4 DNA extraction protocol

DNA was extracted from dry blood samples using Chelex 100 chelating resin (Chelex 100 Resin, iron form, 100 g Bio-Rad laboratory 142-2825) for qPCR assay. 1 ml of 0.5% saponin (950µl 1*PBS + 50µl 10% Saponin) was add into the micro centrifuge tube containing the blood spot and incubate at 4^0C for at least 1 to 2 nights depending on color change. Day 2 vortex for a few seconds and then centrifuge the mixture at 13,000 rpm for 10 minutes and aspirate the saponin but leave the spot in the micro centrifuge tube. Add 1 ml of phosphate buffered saline into the micro centrifuge, close, vortex, and incubate at 4^0C for 30 minutes after that centrifuge at 13,000 rpm for 5 minutes and then remove the PBS as much as possible. Pipette and add 150µL of 30%

Chelex resin solution close the tube vortex and incubate at 95^0C for 12 minutes using a heat block/ shaker. It is important opening cap after 2 min and vortex every 3 minutes, to release pressure. To avoid Chelex Resin Beads in final extract spin for 8 minutes at 13,000 rpm. Transfer the final extract approximately 125µL to a new micro centrifuge and then store box at -20^0C deep fridges. (60) (Details on annex 7)

4.7.5 qPCR Assay protocol

The qPCR assay was run targeting 18S rDNA gene of the parasite and based on using established protocol (61, 62). utilizing 2 species-specific primers (forward: 5'-AGTCATCTTTCGAGGTGACTTTTAGATTGCT-3'; reverse: 5' GCCGCAAGCTCCACGCCTGGTGGTGTC-3') specific to *P. falciparum* and (forward: 5' GAATTTTCTCTTCGGAGTTTATTCTTAGATTGC-3'; reverse: 5' GCCGCAAGCTCCACGCCTGGTGGTGC-3') specific to *P. vivax* that targeted the 18S rRNA genes in a different reaction cocktail. Therefore, the qPCR assay was performed to detect and quantify *P. falciparum* and *P. vivax* only. Amplification was conducted in a 20 µl reaction mixture containing 2 µl of genomic DNA, 10 µl 2 × SYBR Green qPCR Master Mix (Thermo Scientific), and 0.5 µM primer. The reactions was performed in Quantum Studio 3, Applied Bio systems, CA Real-Time PCR Instrument. The reaction conditions were as follows; an initial denaturation at 95°C for 3 min, followed by 45 cycles at 94°C for 30 sec, 55°C for 30 sec, and 68°C for 1 min with a final 95°C for 10 sec. This was followed by a melting curve step of temperature ranging from 65°C to 95°C with 0.5°C increments to determine the melting temperature of each amplified product (Annex 9). Each assay included positive controls of both *P. falciparum*7G8 (MRA-926) and HB3 (MRA-155) isolates as well as *P. vivax* Pakchong (MRA-342G) and Nicaragua (MRA-340G) isolates, in addition to negative controls, nuclease free water. A standard curve was produced from a ten-fold dilution series of the control plasmids (*P. falciparum* and *P. vivax*) and laboratory culture (*P. falciparum*) ranging from 1 to 1.75 × 10−12% parasitaemia to determine the efficiency and detection limit of the qPCR. Samples yielding Ct values higher than 37 (as indicated in the negative controls) was considered negative for *Plasmodium* species. The amount of parasite density in a sample was quantified by converting the threshold cycle (Ct) into gene copy number (GCN) using the follow equation: GCN sample = 2 E×(40-Ctsample); where GCN stands for gene copy number, Ct for the threshold cycle of the sample, and E for amplification efficiency. The differences in the log-

transformed parasite GCN between microscope positive Pf and Pv as well as between G6PD normal and deficient samples was assessed for significance at the level of $P<0.05$ by one-tailed t-tests. (61, 62) (Details on annex 8)

4.7.6 G6PD test protocol

The G6PD test was performed using careSTART™ POCT S1 (G6PD Biosensor and Hb Biosensor combined system). The device was run on power supply in air-conditioned laboratory conditions; testing strips was kept at room temperature in the same laboratory. Blood samples were feed by using precision pipettes; manual instructions was followed. The machine analyze G6PD activity from blood sample drawn directly the finger prick. Results was displayed within five minutes and could be printed or saved electronically. (See annex 9)

4.8 Data analysis

The data were checked for consistency and completeness. After checking the data for completeness, missing values, and coding of the questionnaires, the data were entered in to Microsoft excel and analyzed using SPSS software package version 21.0. Descriptive statistics was employed to summarize the socio-demographic characteristics of the study participants. Bivariate logistic regression was used to determine association of malaria infection with independent variables. Variables with p-value less than 0.25 by the bivariate analysis were candidates for multivariable logistic regression. Odds ratio and the corresponding 95% CI was calculated to determine the strength of the association. P-value < 0.05 was considered significant during the analysis.

4.9 Data quality control

Standard operating procedures were followed strictly. Quality control for G6PD assay was included during running each test. The tests was conducted following the manufacturers' instruction. In general, the quality of qPCR and extraction kit was checked by using known positive and negative control. A positive control slides gives information on the integrity of the staining dye, those blood films positive for *Plasmodium* parasites and 10% of the negative slides was subsequently read by blinded senior lab technologist at Jimma University TIDRC research laboratory.

4.10 Ethical considerations

The study protocol was reviewed and approved by Institutional Review Board (IRB) of Institute of Health, Jimma University **(Ref. No. IHRPGD/711/20)** and permission to conduct the study was also obtained from Bonga Zone Health Department. Patients found positive for malaria parasite were treated at the hospital according to the Ethiopian national malaria treatment guideline (FMoH 2012).

Confidentiality of individual patients' information was maintained during data collection, analysis, and interpretation. After a brief description of the background and aim of the study and the possible benefits and related risks to the study participants, a written consent and ascent were sought from each study subjects or guardian in case of minors.

CHAPTER FIVE

5. Results

5.1 Demographic characteristics of the study participants

Table 1 shows the socio-demographic characteristics of the study participants. A total of 384 study participants were included in the study. Of these 148 (39.7%) were males and 224 (60.2%) were females. The age of the study participants ranged from 4 to 80 years (mean age = 30 years). Of the total number of study participants 347 (93.2%) were literate and 25 (6.7%) were illiterate. Majority of (62.6%) the study participants were farmer. Only 9 (2.4) participants stayed at the current residential address less than 3 months while 365 (97.6%) were permanent residents. 117 (31.4%) participants used ITN the previous night and 255 (68.5%) did not use ITN. Only one subjects was pregnant during the time of survey.

A total of 384 febrile cases were recruited in this study and 372 study participants provided samples for blood film examination, molecular analysis and G6PD test. Demographic, G6PD and microscopic data were obtained from these participants to compare with the molecular results. The remaining case had excluded by exclusion criteria non-optimal blood specimens (insufficient samples) not willing to give consent, chronic patients were blood samples could not be taken. Thus, all analyses were performed based on these 372 individuals.

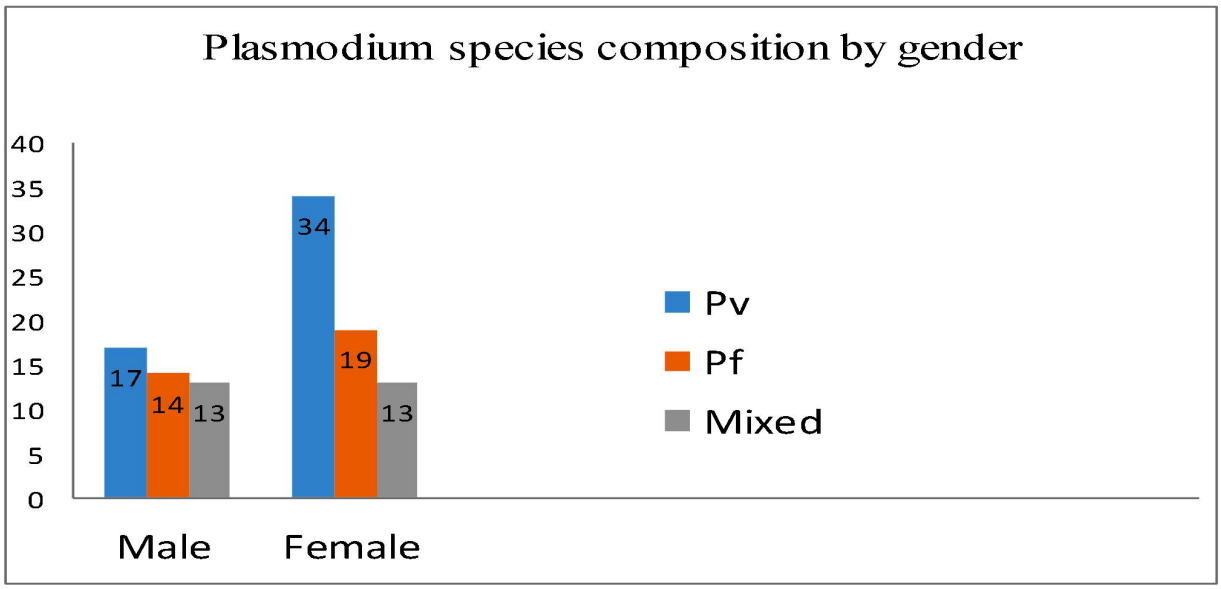
Characteristics		**Participants n (%)**
Sex		
	Male	148 (39.7)
	Female	224 (60.2)
Age		
	<5	1 (0.8%)
	5-15	3 (59.1%)
	>15	368 (23.7%)
Education		
	Not attending school	25 (6.7)
	Literate	347 (93.3)
Occupation		
	Farmer	233 (62.6)
	Others	139 (37.4)
How long have you been stay at the current residential address		
	<3 months	9 (2.4)
	Permanent resident	363 (97.6)
Did you use ITN the previous night?		
	Yes	117 (31.4)
	No	255 (68.6)

Table 1: Demographic characteristics of the study participants in Gebretsadiek Shawo General Hospital, Bonga town, southwest Ethiopia (Jul to Oct, 2020).

5.2 Demographic characteristics of clinical malaria cases

Of the 372 study participants diagnosed for malaria, 110 (29.5%) had malaria based on quantitative PCR assay. On this 44 (29.7%) males and 66 (29.5%) females had malaria infections. The proportion of malaria infection due to *P. vivax, P. falciparum* and mixed infection was 51 (13.7%), 33 (8.9%) and 26 (7%), respectively. In males, the proportion of infection due to *P. vivax*, P. *falciparum* and mixed infection was 17 (11.5%), 14 (9.5%) and 13 (8.8%), respectively whereas, in females, infection due to *P. vivax* was followed by *P. falciparum* and mixed infection 34 (15.2%), 19 (8.5%) and 13 (5.8%), respectively. Although *Plasmodium* infection was recorded in all age groups, a relatively higher *Plasmodium* infection was recorded among cases 5-15 years old (2 out of 3; 66.6%), followed by >15 years old (107 out of 368; 29.1%). Of the total malaria positive cases, nearly 30.1% of the infection were farmers and followed by unemployed, 29.4% among other. One hundred seventeen (31.4%) out of the 372 febrile patients used ITN. Of those who slept under ITN the previous night, the prevalence of malaria was 34.2% and among those who did not use ITN prevalence of malaria infection was 27.5%. Of the total 121 previously *Plasmodium* infected individuals 12.4%, 9.1% and 9.1% were positive for *P. vivax, P. falciparum* and mixed infections, respectively. (Table 3).

Figure 2: Plasmodium species composition by gender using qPCR in Gebretsadiek Shawo General Hospital, Bonga town, Southwest Ethiopia (Jul to Oct, 2020).



5.3 Risk factor of *Plasmodium* infection

Table 2 shows result of logistic regression analysis to determine predictors of *Plasmodium* infection. The variables which were considered for binary logistic regression model included sex, age, educational status, occupation, ITN utilization and previous history of malaria illness. The result of the analysis indicated that all variables did not show significant association with *Plasmodium* infection ($p > 0.05$).

Table 2: Results of bivariate and multivariate logistic regression analysis to determine main predictors of malaria parasite infection at Gebretsadiek Shawo General Hospital, Bonga town, southwest Ethiopia, (Jul to Oct, 2020).

Risk factor	# of samples (% qPCR +ve)	COR (95% CI)	P-value	AOR (95% CI)	P-value
Sex					
Male	148 (29.7)	1.01 (0.64-1.60)	0.964	1.04 (0.66-1.65)	0.877
Female	224 (29.5)	Ref		Ref	
Age	372 (29.6)	1.00 (0.99-1.02)	0.727	1.01 (0.99-1.02)	0.621
Educational status					
Not attending school	25 (36.0)	1.37 (0.57-3.20)	0.467	1.49 (0.62-3.56)	0.342
Literate	347 (29.5)	Ref		Ref	
Occupation					
Farmer	249 (27.3)	0.73 (0.46-1.15)	0.175	0.75 (0.45-1.23)	0.227
Other	123 (34.1)	Ref		Ref	
ITN utilization					
No	255 (34.2)	0.73 (0.46-1.17)	0.187	0.79 (0.48-1.30)	0.383
Yes	117 (27.5)	Ref		Ref	
Previous history of malaria illness					
Yes	121 (30.6)	1.07 (0.67-1.72)	0.767	1.06 (0.66-1.72)	0.779
No	251 (29.1)	Ref		Ref	

Note:- *Age was considered as continuous variable in the crude odds ratio and adjusted odds ratio*

5.4 Sensitivity and specificity of microscopy compared to qPCR.

Using qPCR as a gold standard, the sensitivity and specificity of microscopy was 18.2% and 97.7% respectively. The corresponding positive and negative predictive values were 77% and 74%, respectively.

Table 3: Sensitivity and specificity of microscopy against qPCR, detection of *Plasmodium* infection in Gebretsadiek Shawo General Hospital, Bonga town, southwest Ethiopia (Jul to Oct, 2020).

Microscopy	**qPCR**		**Total**
	Positive n (%)	**Negative n (%)**	**n (%)**
Positive n (%)	20	6	26
Negative n (%)	90	256	346
Total n (%)	110	262	372

Of the total 110 (29.6%) *Plasmodium* positive samples by qPCR, microscopy detected only 26 (7%). Of the total 346 negative by microscopy, qPCR detected additional 84 cases. With respect to each species, qPCR detected a total of 51 *P. vivax* cases while microscopy detected 3 of the *P. vivax* cases. Moreover, only 7 *P. falciparum* and 1 of the mixed infections were detected by microscopy.

Table 4: Microscopy vs qPCR for detection of *plasmodium* species in Gebretsadiek Shawo General Hospital, Bonga town, southwest Ethiopia (Jul to Oct, 2020).

Microscopy	**qPCR**				**# Total**
	# *Pv positive*	**# *Pf positive***	**# Mix *positive***	**# Negative**	
# *Pv positive*	3	2	0	2	7
# *Pf positive*	4	7	3	4	18
# Mix *positive*	0	0	1	0	1
# Negative	44	24	22	256	346
Total n	51	33	26	262	372

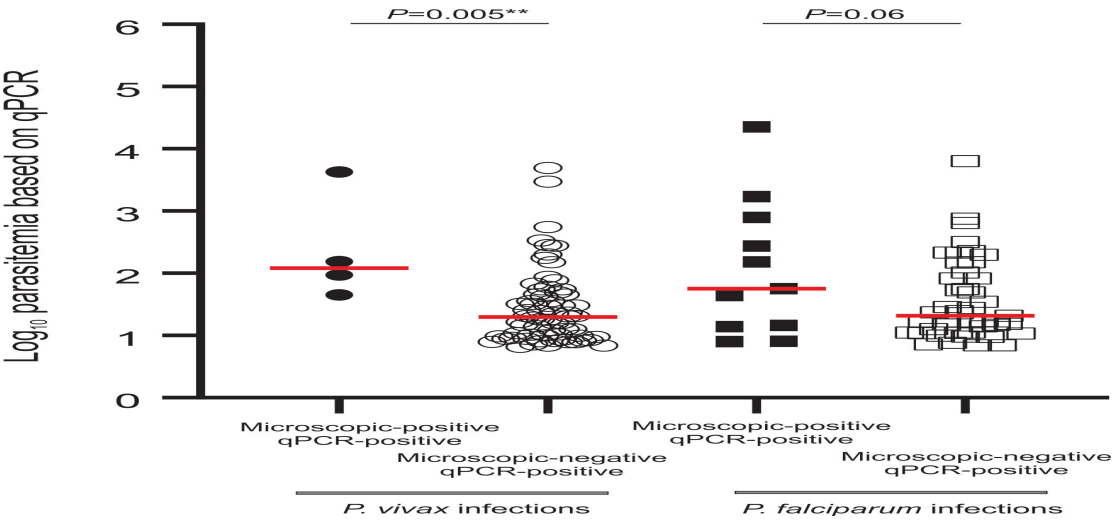


Figure 3: Comparison of Plasmodium species parasite density using qPCR among P. vivax and P. falciparum positive cases in Gebretsadiek Shawo General Hospital, Bonga town, southwest Ethiopia (Jul to Oct, 2020).

With qPCR as the reference for malaria diagnosis, overall, the agreement between the results for microscopy and qPCR assay was low. Calculating correlation between smear parasite density and the CT value in the *Plasmodium* species qPCR assay is difficult but the odds of *P. vivax* by qPCR was 2.5(95% Cl: 1.0; 6.4, $p < 0.005$) times more positive than microscopy.

5.5 G6PD prevalence

Of the 372 study subjects tested for G6PD, 56(15.1%) were G6PD deficient, 53(14.2%) had intermediate and 263(70.7%) are normal. The prevalence of G6PDd in males was 31 out of 56 (16.5%) and 25 out of 56 (14.2%) in females. There was a variation in the rate of parasite infection between sexes with G6PD; Among G6PD deficient patients, parasite infection in males was 36% and 32.2% in females; while among G6PD normal individuals, prevalence of parasite infection was 28.4% in males and 27.8% in females. In G6PD deficient patients, the prevalence of *P. vivax, P. falciparum and* mixed infection was 8(14.3%), 5(8.9%) and 6(10.7%), respectively. In G6PD normal individuals, the prevalence of *P. vivax, P. falciparum and* mixed infection was 33(12.5%), 25(9.5%) and 16(6.1%), respectively. There is no significant difference in G6PD enzyme activity levels between *P. vivax* and *P. falciparum* infected individuals (Table 5).

Table 5: Results of G6PD test using qPCR among study participants in Gebretsadiek Shawo General Hospital, Bonga town, southwest Ethiopia (Jul to Oct, 2020).

		QPCR		
G6PD level	**# tested n(%)**	***P. Vivax* n(%)**	***P. Falciparum* n (%)**	**Mixed infection n(%)**
Normal	263 (70.7%)	33 (12.5%)	25 (9.5%)	16 (6.1%)
Intermediate (for Female)	53 (14.2%)	10 (18.9%)	3 (5.7%)	4 (7.5%)
Deficient	56 (15.1%)	8 (14.3%)	5 (8.9%)	6 (10.7%)

In G6PD deficient patients, the prevalence of *Plasmodium* infection was 19 (33.9%) whereas, in G6PD normal, it was 74 (28.1%). There was no significant difference in *P. vivax* and *P. falciparum* parasite density between the G6PD normal and G6PD deficient individuals (Figure 4), Moreover, there is no significant difference, in G6PD enzyme activity levels between *P. vivax* and *P. falciparum* infected individuals' $p > 0.05$ (Figure 4).

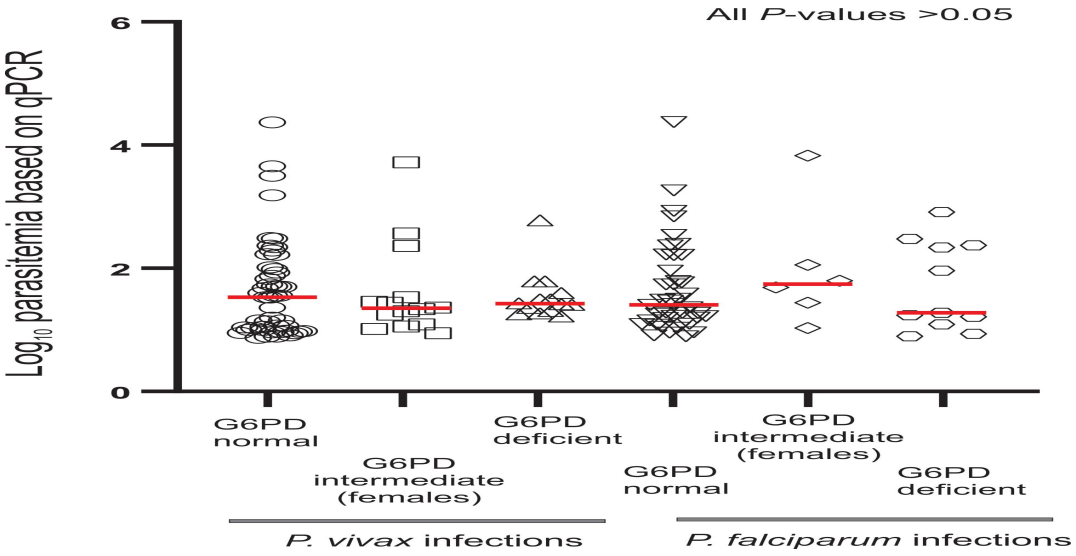


Figure 4: Plasmodium parasite density estimated by qPCR among G6PD normal, intermediate, and deficient patients in Gebretsadiek Shawo General Hospital, Bonga town, southwest Ethiopia (Jul to Oct, 2020).

CHAPTER SIX

6. DISCUSSION

The Current study employed a facility based cross-sectional study of submicroscopic malaria. The results showed that of all the study subjects, qPCR detected 29.6% while 7% of the cases were positive by microscope. These findings show that submicroscopic malaria infection was high among the study subjects. Given the seasonal nature of malaria transmission of the study area, this finding has an important implication for malaria elimination in the area. As the malaria patients were not treated with anti-malarial drugs, they may serve as reservoirs of infection in the community and when they travel to malaria-free areas. Transmission of malaria associated with submicroscopic malaria infection has been documented earlier elsewhere (45)

The overall submicroscopic malaria prevalence in this study was comparable with findings of studies done in Jazan, southwestern Saudi Arabia and Dielmo, Ndiop Senegalese villages where the prevalence of submicroscopic infection 23.3% and 26.6% were reported, respectively. (28, 42, 43) Another study conducted in five low endemic settings in Ethiopia shows the wide-scale presence of submicroscopic parasitaemia (0.7%) were detected by microscopy, whereas 12.7% positive by qPCR and another study in Malo, southwest Ethiopia the prevalence of submicroscopic was 9.7% which is lower than this study (62). In contrast, the prevalence of submicroscopic malaria infections in this study was less than from the study conducted in Tororo District, Ugandan, in which prevalence of 39.2% (44).

In this study, males had slightly more clinical malaria than females. The reason for this was unclear, but it is possible that gender impacts on the risk of malaria infection, as well as their ability to access services, social, economic and cultural factors play a crucial role in determining differences in gender-related vulnerability to malaria infection and access to malaria prevention and treatment services for example males travel to work in the field and may spend longer time outdoor for farming activities during the evening and thus exposed more frequent to mosquito bite.

Antimalarial drug treatment is one of the most widely used malaria control methods utilized in malaria-endemic countries. The aims of treatment is to prevent death or long-term morbidity and debilitation from malaria, to treat an acute episode of illness, and to clear the infection entirely so

that it does not reoccur. Without properly taking antimalarial drug or effective treatment, the number of parasites will increase with every 2-day cycle of reproduction. A mature infection may involve up to 1012 circulating plasmodia. (58) It is not surprising that if the patients did not take their antimalarial properly the relapse will arising from persistent liver stages of hypnozoites. (60). In this study 12.4% *P. vivax* infections had a history of malaria illness and this posed a risk for *P. vivax* infection. This was likely due to the relapse of the vivax parasite arising from persistent liver stages of hypnozoites (64). In Ethiopia, an estimated 40% of infected cases detected during the main peak transmission season was attributed to relapses (66). In Southeast Asia, *P. vivax* relapse accounts for approximately 30% of the infected cases (65).

Additionally, this study documents substantial discordance between microscopic and molecular diagnoses of malaria. This could be partly due to a significantly lower parasitemia in the submicroscopic samples as shown in the *P. vivax* cases (Figure 3). Even the qPCR-based parasite density in microscopic-negative samples was very low than the microscopic-positive samples, despite differences in sample size. Therefore, the author believed that these findings reinforce both the feasibility and the necessity of incorporating molecular diagnostics for malaria in clinical studies.

According to a systematic review of *Plasmodium infection* prevalence measured by both microscopy and PCR, the prevalence of infection measured by microscopy was, on average, 50.8% of that measured by PCR. Similarly, in Kenya only 68% of real-time PCR-positive samples were detected by microscopy. This substantial discordance highlights the unreliability of the blood smear for malaria diagnosis particularly in low transmission settings. Studies suggested that conventional methods miss a substantial proportion of *Plasmodium* infections in surveys of endemic populations, especially in areas with low transmission of infection. Declines in parasite prevalence in areas where malaria is endemic may further compromise the positive predictive value of microscopy and limit the utility of microscopy for case detection in clinical studies (46).

In the current study, mixed infections were detected by microscope which was 3.8% and 23.6% by qPCR., almost all of which were misdiagnosed. This result is comparable to study conducted in Western Thailand where 90.2% of malaria infections were submicroscopic, while mixed infections accounted for 68% of cases (48).

The present study G6PDd prevalence was 15.1%; higher than the ranges reported in southwest Ethiopia (7.3%) (39). This figure was relatively lower compared to the WHO report from parts of Africa which was as high as 35% (70). The highest prevalence rates are found in Africa, the Middle East, certain parts of the Mediterranean, and certain areas in Asia. In these regions, the rate ranges from 5% to 30% of the population. The severity of G6PD deficiency can vary based on specific racial groups. The severe form of the disorders occurs more often in the Mediterranean Region (71). The prevalence of G6PDd significantly varied among different countries and regions or even localities, for instance, the report of 4% G6PDd in Gambella, 0.8% in Oromiya, and 0.5% in Benishangule Gumuz, respectively. There are several reasons for such differences. In lowland areas G6PD deficiency prevalence ranged from 1.4 to 14.3% in the southwest (Nuer and Anuak ethnic groups) and northeast (Aregoba ethnic group in Afar) regions where certain ethnic groups, are dominant (38, 39). In southern European countries, such as Greece and Italy, G6PDd prevalence ranged from 1-30% and 0-3% respectively. (37) G6PDd prevalence in Yemen, Cameron and Saudi Arabia was 7.9%, 7.1%, 9.1%, respectively (42, 72, 73). The variation in the prevalence of G6PDd could be attributed to ethnic differences.

In terms of gender, although the frequency of G6PDd was higher in males (16.5%) than in females (14.2%) in the present study, other similar studies detected (35.6%) among the male participants was higher than that found in the female participants (13.33%). This finding is similar to that of Jacques et al. (2007) who reported 20.5% and 12.3% G6PD prevalence in males and females, respectively (75).The occurrence of more defects in males is due to the fact that the males are hemizygous whilst females are dizygous for the X chromosome. (17, 18). Furthermore, the difference in G6PD enzymatic activity between male and female could be due to the fact that the phenomenon of inactivates only one X chromosome in each female's cell (76, 77). A significant difference between males and females typical for heterozygous females who exhibit variable expression of G6PD activity. This may be due to the differences in ethnic group composition, genotype and detection methods between these regions. However, the present study study was not able to detect such differences.

In G6PD deficient patients, the prevalence of *Plasmodium* infection was 19 (33.9%), compared to the G6PD normal of which the prevalence of *Plasmodium* infections was 74 (28.1%). Findings of this study showed that the variation in G6PD level had no effect on the number of clinical malaria cases nor the parasite density. The mechanism underlying this pattern is not

known. Previous study suggested that low level of G6PD may increase sensitivity of *P. vivax* to oxidative stress (70). As G6PD deficiency leads to increased oxidative stress in red blood cells, this may in turn have a influence on the parasite. As such, individuals who possess specific mutation are expected to have protection against malaria.

Further, erythrocytes are also the most affected cells from G6PD deficiency. This situation also suggests the relationship between malaria and G6PDd. In several studies, it was demonstrated that G6PD deficiency provides a protection against malaria infections. In one of the early studies, it was indicated that *P. vivax* parasites preferred to invade younger erythrocytes (78, 79), which possessed high levels of G6PD enzyme. Since enzyme levels are diminished in older erythrocytes, parasites do not prefer to invade these erythrocytes. These studies suggested the protective effect of G6PD deficiency from parasitemia (80, 81). Recently, Ruwando et al. also indicated African form of G6PD deficiency decreased at a rate of 46% to 58% of selective advantage of resistance to malaria was counterbalanced with selective disadvantageous results of G6PD deficiency, and this stopped the rise of malaria frequencies in endemic regions (82). In another study, Ninokata et al. (2006) investigated 345 healthy adults and found out that 10% of these individuals had G6PD deficiency. Interestingly, it was observed that none of the individuals had molecular evidence of malaria infection. According to this study, researchers postulated that G6PD deficiency provided an advantageous genetic trait against malaria (83). The exact mechanism of this protection is still unknown. However, this study did not provide support to this phenomenon.

CHAPTER SEVEN

7.1. Conclusion

This study shows higher prevalence of submicroscopic infections among individuals visiting the health facilities. In the other hand, this study highlights the relative sensitivity and specificity of microscopy vs qPCR detection of *plasmodium* infections, microscopy showed less sensitivity and specificity than qPCR sensitivity and specificity. Based on G6PD phenotyping, the present study revealed a modest prevalence of G6PD deficiency among febrile patients and there is no significant difference in G6PD enzyme activity levels between *P. vivax* and *P. falciparum* infected individuals.

7.2. Recommendation

Based on the findings, the authors of this study are therefore, recommend to implement advanced techniques to detect submicroscopic malaria infections to protect on risk group and create awareness about G6PD and a routine testing for G6PD status prior to administrating primaquine for radical cure of *Plasmodium* infected patients in Bonga area Ethiopia.

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Annexes

Annex 1. Information sheet and informed consent English version

Study title: Prevalence of sub-microscopic malaria infections among febrile individuals in Bonga town, South West Ethiopia

Name of Participant:_____**Age:** _____years

Each volunteer was given a copy of the full Informed Consent Form.

Investigator conducting this study: Beka Raya, School of Medical Laboratory Sciences, Institute of Health, Jimma University, Jimma, Ethiopia; Tel: +251 917 250436, Email: bekara08@gmail.com.

Sponsor: self

Purpose of this study: We want to examine sub-microscopic malaria infection among febrile individuals in Bonga town. This information will help us determine how to improve malaria prevention and control in this area in particular and Ethiopia in general.

Procedures to be followed:

1) We will gather some personal information such as age, gender, occupation, travel history, bed-net use and malaria history in order to document how malaria occurs in your local community.
2) For malaria diagnosis, the patients are normally required to provide two drops of blood on slide so that the malaria parasite can be observed under a microscope. For more accurate diagnosis, we would like to obtain an additional blood samples for qPCR and blood film preparation to double check the accuracy of the diagnosis.
3) The blood was analyzed for G6PD deficiency by Biosensors machine.

For this purpose, a 200µl of blood sample was enough.

Duration of the study: The study was conducted for a period of four month.

Inclusion criteria: We will include volunteers who are willing to participate in the study and have no reported chronic or acute illness, regardless of their sex, and economic status, with prevision of informed consent, and older than 6 months at recruit.

Exclusion criteria: People who are unwilling to participate in the study or have planned to move out of the study area was excluded.

Discomforts and risks: The finger pricked blood collection method causes slight discomfort. Sterile lancet (with sterile ethanol) was used for every single person, the procedures will pose no risk of being infected by other pathogens.

Benefit to participants: You will not receive financial benefit from participation; however, the project will cover malaria and G6PD diagnosis costs for those patients coming to the health facility and participated in the study. Moreover, your participation in this research will help us obtain more information about submicroscopic malaria infections, which is useful for development of more effective prevention and control measures in the future

Confidentiality: Information related to you or your child was kept confidential to the extent provided by law. Your or your child's identity was coded and will not be associated with any published results.

Right to refuse and withdraw: Your or your child's participation in this study is voluntary and you may discontinue your or your child's participation at any time without prejudice and without affecting future health care. You are not obliged to respond to all questions in the questionnaire.A decision not to participate in the study will have no impact on your access to health care provided by the government.

Use of the blood samples collected: Samples was used only for science. No names will ever be disclosed and data was kept very confidential. I understand that a small portion of the samples was stored for future testing. The nature of this testing is not known at this time; however, investigators may look at inherited factors which are related to diseases by examining DNA obtained from the stored samples. By signing this form, I understand that I am giving consent for any future studies of genes that we may perform in the laboratory. The DNA sample will remain the property of Jimma University, and may be shared with other researchers as long as confidentiality is maintained. All names was removed from samples prior to being given to other researchers. The DNA samples are stored and tested with an identifying code number, and your name will not appear on the stored samples. I will not be told of these possible tests, nor will I receive results of any of these tests. I understand that there is a possibility that the DNA samples, which I am providing under this study, may also be used in other research studies and could potentially have commercial applicability. I understand that data are stored at centralized data management facility at the Jimma University for researchers to analyze malaria burden changes

and for future genetic analysis of malaria parasite. I will not receive any revenues generated by commercial application. Results of studies may be reported in medical journals or at scientific conferences or meetings. However, individuals in the study will not be identified in any way.

New findings: You was told of any significant new findings developed during the course of this study.

Parties that may review research records: Jimma University Faculty of Health Sciences Institutional Review Board (IRB), Jimma, Ethiopia may review the research protocol.

Consent form: The consent form was explained to each study subjects and signed by the Investigators or the leading scientists conducting the study.

I have been invited to participate in research project on malaria. I understand that it will involve by giving finger pricked blood 200µl. I have been informed that the risks are minimal. I have also been assured that all my personal identities was kept confidential. I am aware that there may be no immediate benefits to me personally. I have been provided with the name of the researchers, who can be easily contacted using their number and address I was given.

I have read the foregoing information, or it has been read to me. I have had the opportunity to ask questions about it and any questions that I have asked have been answered to my satisfaction. I consent voluntarily to participate as a subjects ***in this research and understand that I have the right to withdraw from the research at any time without in any way affecting my medical care.***

Print Name of Participant_____ Finger print if illiterate

Signature of subjects _____ Date _____

Witness' Name _____ Witness' Signature _____ Date _____

Researcher's Name_____ Researcher's Signature _____Date_____

A copy of this Informed Consent Form has been provided to subjects (initialed by the researcher/assistant)

Annex 2. Information sheet and informed consent amharic version **(ቅፅ : በጥናቱ ላይ የመሳተፍ የስምምነት ቅፅ)**

<u>የወባ መረጃን ከጤና ተቋማት ለመሰብሰብ</u>

የጥናቱ ርዕስ: በሞሎኪውላር መንገድ የወባ በሽታ ስርጭትን ለማጥናት በቦንጋ ገ/ጻዲቅ ሸዋ አጠቃላይ ሆስፒታል, ኢትዬጵያ *(*Prevalence of sub-microscopic malaria infections among febrile individuals in Bonga town, South West Ethiopia*)*

የተሳታፊዉ ስም:_____ **እድሜ:** _____ዓመት

የዚህ የስምምነት ቅፅ ግልባጭ ለሁሉም ተሳታፊዎች የሚሰጥ ነዉ

1.የተመራማሪ ስም: ቤካ ራያ, ጅማ ዩኒቨርሲቲ የህክምና ላቦራቶሪ ት/ት ከፍል፣ጅማ፣ እትዮጵያ፤ስልክ: +25191725 04 36፣Email: bekara08@gmail.com

2. ስፖንሰር: በግል

3.የጥናቱ አላማ: በሞሎኪውላር መንገድ የወባ በሽታ ስርጭትን ለማጥናት ነዉ። ከዚህ የሚገኘውም መረጃ በቦታው እና በአጠቃላይ በኢትዮጵያ የወባ ቁጥጥርን ለማሻሻል ይረዳል።

4.የሚሰሩ ስራዎች በቅደም ተከተል:

1) በህበረተሰቡ ዉስጥ የዎባ በሽታ እንዴት እንደሚከሰት ለማጥናት የተሳታፊዎችን እድሜ፣ፆታ፣ ስራ፣የጉዞ ታሪክ፣ የአጎበር አጠቃቀምና ከዚህ በፊት በዎባ ስለመያዛቸዉ መረጃ እንሰበስባለን።
2) የወባ በሽታን ለመመርመር ይረዳ ዘንድ ከተሳታፊዎቹ (ከህመሚን) የደም ናሙና ይወሰዳል። የወባ በሽታ አምጪ ተዋሲያን በማይክሮስኮፕ የሚታዩ ሲሆን፤ ለምርመራዉ ጥራት ተጨማሪ የደም ናሙናም የሚወሰድ ይሆናል። ለዚህ ስራ ጥቂት ደም ማለትም 200ማሊ በቂ ነዉ፡
3) በተጨማሪም የሚወሰደዉ ደም በረቀቀ መንገድ ወባን ለመመርመር ይሆናል

5.የጥናቱ ጊዜ: ምርምሩ ለ4 ወር የሚሰራ ይሆናል።

6. ምርምሩ ላይ የሚያሳትፉ መስፈርቶች: በጥናቱ ላይ ለመሳተፍ ፍቃደኛ የሆኑና ሌላ በሽታ የሌለባቸዉ፣ ጾታና የሓብት ሁኔታ ሳይለይ ሁሉም እድሜያቸዉ ከ 6 ወር በላይ ያሉት መሳተፍ ይችላሉ።

7. **ምርምሩ ላይ የማያሳትፉ መስፈርቶች:** በጥናቱ ላይ ለመሳተፍ ፍቃደኛ ያልሆኑና ጥናቱ ከሚሰራበት አካባቢ ለመልቀቅ እቅድ ያላቸዉ አይካተቱም

8. **ጉዳትና ተጋላጭነት:** የደም ናሙና በሚወሰድበት ግዜ ትንሽ የህመም ስሜት ሊኖር ይችላል። ደሙ የሚወሰደዉ በንፁህ መሳርያ ስለሆነ ለየትኛዉም ተላላፊ በሽታ አያጋልጥም።

9. **ጥቅማጥቅም:** በምርምሩ ላይ በመሳተፍዎ ምንም የገንዘብ ጥቅም አያገኙም። ነገር ግን ለምርመራው የሚያስፈልገውን ወጭ ሁሉንም በፕሮጀክቱ የሚቨፈን ሲሆን በተጨማሪም ከጥናቱ የሚገኘዉ መረጃ ለወደፊቱ የወባ በሽታን ለመቆጣጠር ይጠቅማል።.

10. **ምስጥራዊነት:** ማንኛዉም ከእርስዎ ወይም ከልጆ የሚሰበሰብ መረጃ ሚስጢራዊነቱ የሚጠበቅ ይሆናል።የርስዎ ወይም የልጅዎ በኮድ የሚፃፍ ሲሆን ስማችሁ የትኛዉም ህትመት ላይ አይኖርም።

11. **በጥናቱ ላይ ያለመሳተፍ እና የማቋረጥ መብት:** የርስዎ ወይም የልጅዎ ተሳትፎ በፍቃደኝነት ላይ ብቻ የተመሰረተ ሲሆን በየትኛዉም ሰዓት ማቋረጥ ወይንም ልጅዎ እንዲያቋርጥ ማድረግ ይችላሉ።በቃለመጠየቅ ግዜ ለሁሉም ጥያቄዎች መልስ እንዲሰጡም አይገደዱም። በጥናቱ ላይ ባለመሳተፍዎ የሚቋረጥቦት የጤና አገልግሎት አይኖርም።

12. **የሚሰበሰበዉ የደም ናሙና ጥቅም:** በደም ዉስጥ የሚገኝ የወባ በሽታ ኣምጪ ተዋሲያን አይነቶች ለማጥናት (ለሳይንሳዊ ምርምር ብቻ) የሚዉል ይሆናል። ለወደፊት ጥናት እንዲያገለግል ትንሽ የደም ናሙና እንደሚቀመጥ አዉቃለሁኝ። ይህንን የስምምነት ቅፅ የምፈርመዉ የደም ናሙናዉ ለወደፊት ጥናት ሊዉል እንደሚችል እና ከሚመረቱ ውጤቶች (commercial application) ሊኖሩት እንደሚችሉ በመረዳትም ጭምር ነዉ። የደም ናሙናዉ የጅማ ዩኒቨርሲቲ ንብረት ሲሆን ለምርምር ጉዳይ ለሌሎች ተመራማሪዎችም ከፊሉ የሚሰጥ ይሆናል። የደም ናሙናዉ በጅማ ዩኒቨርሲቲ የሚቀመጥና ለወደፊት ወባ ላይ ለሚደረገዉም ምርምር የሚዉል መሆኑንም ተረድቻለሁ። በናሙናዉ ላይ ለወደፊት የሚደረገዉም ምርመራ እኔን ሳየሳዉቁኝ እንደሚሆንም እና ከምርሩም ሊገኝ ከሚችል ገንዘብ ተካፋይ እንደማልሆን ተረድቻለሁ። ናሙናዉ የሚቀመጠዉ በእኔ ስም ሳይሆን በኮድ እንደሆነ ተነግሮኛል።። ከጥናቱ የሚገኝ ዉጤት በህክምና ጆርናሎች ወይም ኮንፈረንሶች ላይ ሪፖረት ሊደረግ ወይም ሊታተም ይችላል። ነገርግን የግለሰቦች ስም አብሮ አየወጣም።

13. **አዲስ ግኝት:** በጥናቱ ሂደት አዲስ ግኝት ከተገኘ ለርስዎ የሚገለፅ ይሆናል

14. **ምርምሩን የሚገመግሙት ተቋማት:** የጅማ ዩኒቨርሲቲ ጥናቱን ይገመግሙታል።

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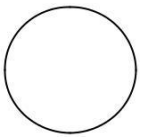
15. **የስምምነት ቅፅ**፤የስምምነት ቅፁ ለሁሉም ተሳታፊዎች የሚገለፅና በተመራማሪዎቹ የሚፈረም የሆናል።

በወባ ላይ በአካባቢያችን በሚሰራዉ ምርምር ላይ እንዲሳተፍ ተጋብዣለሁኝ። በጥናቱ ግዜም ከጣቴ ላይ የደም ናሙና እንደሚወሰድም ተነግሮኛል።በመሳተፌ ምክንያት ሊደርስብኝ የሚችለዉ ተጋላጭነት ትንሽ እንደሆነ ተነግሮኛል። ማንኛዉም ከእኔ የሚወሰድ መረጃ ምስጥራዊነቱ እንደሚጠበቅ ተነግሮኛል። በጥናቱ ላይ በመሳተፌም የተለየ የማገኘዉ ጥቅም እንደሌለም ተነግሮኛል። የተመራማሪዎቹ ስምና አድራሻ የተሰጠኝ ሲሆን ጥያቄ ካለኝ በማንኛዉም ሰዓት ማናገር እንደሚችል ተነግሮኛል።

ከላይ የቀረበዉን መረጃ አንብቤ ተረድቻለሁኝ ወይንም ተነቦልኛል። ግልፅ ስላልሆነልኝ ጉዳዮችም ሲኖሩ ጥያቄ እንድጠይቅ እድል የተሰጠኝ ሲሆን ለጥያቄዎቼም መልስ ተሰቶኛል። ስለዚህ በጥናቱ ላይ የመሳተፍ ፈቃደኝነቴን ስገልፅ በማንኛዉም ሰዓት የማቋረጥ መብቴ የተጠበቀ መሆኑን ይሁም በመሆኑ በመረዳትም ጭምር ነዉ።



የተሳታፊዉ ስም_____ የጣት አሻራ ላልተማሩ

ፊርማ _____ቀን _____

ምስክር _____ የምስክር ፊርማ _____ ቀን _____

የተመራማሪዉ ስም_____ የተመራማሪዉ ፊርማ_____ቀን _____

የዚህ የስምምነት ቅፅ ግልባጭ በተመራማሪዉ አማካይነት ለጥናቱ ተሳታፊ ተሰቷል

Annex 3. Questionnaire

An interviewer guided questionnaire for assessment to plasmodium infection among Bonga town.

Study Site for this research– Bonga town Ethiopia

1. Subjects **Name_____ 2. Survey Date _____ 3a. Case Number_____**

4. Full Name _____

5. Date of Birth or Age _____6c. Gender (M) Male (F) Female

6. How long have you been stay at the current residential address?

(A) 0 ~ 3 Months (B) 4 ~ 6 Months (C) 7 ~ 12 Months (D) 1 ~ 3 Years (E) More than 3 Years E

7a. Do you consider yourself to be migrant worker (stay less than 6 month and travel back to your original place)? (N) No (Y) Yes

7b.Where Did You Come from Originally? **Ethiopia**

Region_____Zone_____Woreda_____Kebele_____Village _____Village

Code or ID number Household Information_____

8. Household head at patient's address? _____ phone number ? _____

9. Ethnicity

(1) Oromo (2) Amhara (3) Anuak (4) Nuer (5) Tigray (6) Gurage (7) Welayta (8) Hadiya (9) Kafficho (10) Dawro (11) Yem (12) Kembata (13) Mezhenger (14) Shakacho (15) SNNP (16) Afar (17) Harari (18) Somali (51) other African 51 (52) Non-Africans (Arabs, Indians, Asians, and Europeans) 52 (99) Others 99

10. Select the primary occupation or job

(1) Agricultural worker (2) Trader (3) Office worker/Teacher (4) Student (5) Non-school child (6) Guardsman/Soldier/Peace officer (7) Herdsman (8) Fisherman (9) Mining (10) Factory/Construction worker (11) Unemployed (99) Others

11. Select the highest education level

(1) Illiteracy (2) Preschool (before school age) (3) 1-8 (4) 9-10 (5) 11-12 (6) College or University (7) Msc holder (99) Others

12. Have you been diagnosed for malaria in the past? If Yes how long?

(X) N/A or No past Malaria (A) 0 ~ 3 Months (B) 4 ~ 6 Months (C) 7 ~ 12 Months (D) 1 ~ 3 Years (E) More than 3 Years (U) Yes, but not sure the date

13. The past diagnosis of malaria parasite species

(0) N/A, Negative, Undetermined, or Not Done (1) *P. falciparum* (2) *P. vivax* (3)Mix-Pv+Pf (9) Uncertain (10) Don't know (forgot)

14. If positive? where have you been treated?

(0) N/A (1) This clinic (2) Other clinic (3) Private practitioner (4) Self-treatment

15. Past Prescription Did you take all the anti-malarial drugs as prescribed?

(X) N/A (N) No (Y) Yes (U) Uncertain

16. Since your fever started, did you take any antimalarial (drug) before going to health facility?

(N) No (Y) Yes (U) Uncertain

17. Temperature (°C) Axillary (under the arm) Temperature

18. Is pregnant?(X) N/A (N) No (Y) Yes

19. How many weeks have you pregnant? _____

20. Symptoms within past 2 days (select all that apply)

(X) None (A) Fever (B) Chills/Shivering (C) Malaise (D) Fatigue (E) Muscle pain (F) Joint pain (G) Headache (H) Irritability (I) Nausea (K) Vomiting (M) Diarrhea (O) Abdomen pains (P) Loss of appetite (R) Breathing difficulty (S) Dizziness (T) Coughing (U) Stomachache (Z) Others

20a. Other Symptom (if applicable) Severe malaria symptom(s) (select all that apply)

(0) None (1) Impaired consciousness (2) Prostration (3) Multiple convulsions (4) Respiratory distress (metabolic acidotic) (5) Circulatory collapse (6) Jaundice (7) Hemoglobinuria (8) Abnormal bleeding (9) Pulmonary edema (radiological) (10) Death (99) Others

20b. What drugs have been prescribed for the patient? (select all that apply)

(0) N/A or No prescription (1) Coartem (2) Other ACT (3) Chloroquine (4) Sulfadoxine-pyrimethamine (5) Quinine (6) Primaquine (7) Don't know (8) Others

22. Hemoglobin (Hb) (unit:g/dL)

23c. Blood glucose (unit:mmol/L)

24. Malaria RDT result: (0) Negative (1) *P. falciparum* (2) *P. vivax* (3) Mix: Pv+Pf

25. Microscopic Diagnosis Result

(0) Negative (1) P. f (2) P. v (3) Mix: Pv+Pf

Survey Staff Hospital/Clinic Staff who filled this form_____

Annex 4. Blood collection and smear preparation

Required materials

Sterile lancet. 70% ethanol alcohol cotton Gloves Safety box Frosted slides Absolute methanol Slide box 300 µl capillary EDTA tube

Procedure

1. Pre-cleaned slides were labeled on frosted part of the slides with patient's identification number name and code of the villages.
2. The tip part of ring finger was cleaned with a cotton swab moistened with 70% alcohol.
3. After being dried, the clean part of the finger was pricked with sterile blood lancet. If blood did not well up, the finger was gently squeezed with thumb and index finger.
4. The first drop of blood was wiped off with clean cotton ball and the second drop was used for smear preparation.
5. Small drop of blood (~10 µl) was placed on microscope slide just before the center of the slide for thin smear and one larger drop of blood (~15 µl) at about the center of the second half of the same slide for thick smear.
6. Thin smear was prepared with spreader slide by placing it in front of the small drop and back to the blood until it spread at the lower edge of the spreader slide and the blood was smeared along the length of the slide by holding the spreader at 30-450 that extended ½ to 2/3s of the total slide area. Thick smear was prepared using the corner of another microscope slide to form a rough circle and an area about 15 X 15 mm was covered evenly. The thickness of the smeared slides was checked by newsprint. So that both thin and thick blood smears were prepared on the same microscope slide.
7. The slides were kept on slide tray at horizontal position to be dried with air in dust free areas and were protected from flies.
8. Only the thin smears were fixed with absolute methyl alcohol for 3-5 seconds in coplin jar.
9. The slides were removed after 3–5 seconds, excess methanol was drained back into the Coplin jar, and placed the slides flat on a clean paper with the smear side up, to remove excess methanol.
10. Each slide was placed horizontally in the slide box in the same row after the methanol evaporated completely.
11. The slides was transported to TIDRC parasitology laboratory.

Annex 5. Giemsa's stain procedure

A. Principle

Giemsa stain is used to differentiate nuclear and/or cytoplasmic morphology of platelets, RBCs, WBCs, and parasites. The most dependable stain for blood parasites, particularly in thick films, is Giemsa stain containing azure B. Liquid stock is available commercially. The stain must be diluted for use with water buffered to pH 6.8 or 7.0 to 7.2, depending on the specific technique used. Either should be tested for proper staining reaction before use. The stock is stable for years, but it must be protected from moisture because the staining reaction is oxidative. Therefore, the oxygen in water will initiate the reaction and ruin the stock stain. The aqueous working dilution of stain is good only for 1 day.

B. Required materials and reagent

Giemsa stain solution Buffer solution Oil immersion Staining trough Binocular Microscope Timer Drying rack

C. Procedure

1. 10% Giemsa working solution was prepared by mixing one hand Giemsa stock solution with 9 hand buffer solution at PH of 7.2 (e.g. 10ml Giemsa's stock solution and 90ml buffer solution) and placed in staining trough.
2. The slides were placed back to back in staining rack with the smears facing outwards and deep in the staining solution.
3. The slides were stained for 10 minutes
4. The slides were removed and rinsed gently with tap water that kept in a dish.
5. The water was drained and the slides were placed on drying rack at vertical position.
6. After being dried, each slide was examined microscopically using a 100x oil immersion lens and 10x eyepieces and the parasite species was identified.
7. When malaria parasites detected in a blood film, the parasite density were determined by counting the number of parasites (asexual form only) present against 200 white blood cells in a thick smear and multiplied by 40 to arrive at an approximate parasite count per microlitre of blood. This was based on the assumption that the average WBC count is 8,000/µl blood. The number of asexual parasites was calculated using the following formula:

$$\text{Parasites}/\mu L = \frac{\text{No of asexual parasites} \times 8000 \text{ leucocytes}}{200 \text{ leucocytes}}$$

Annex 6. DNA extraction by (30% Chelex)

DNA extraction protocol for dried blood sample

Day 1

1. Enter sample info in excel (Extraction list). Specify date of extraction
2. Label 2 sets of 1.5 ml centrifuge tubes and 1 set of 0.5 ml centrifuge tubes with number that match precisely with the filter paper sample ID
3. Cut 1 piece of approximately 5*5mm^2 (normally half or a quarter of the dot depending on the size) of blotted filter paper. Cut in small strips before put in a 1.5ml tube
4. Add 1ml of 1*PBS containing 0.5 % Saponin (950ul 1*PBS + 50ul 10% Saponin)
   - Make a cocktail. For 13 mL: 12,350 µL PBS and 650 µL Saponin.
5. Mix well and make sure filter papers are completely immersed inside buffer
6. Incubate at 4C for 1- 2 nights depending on color change

Day 2

1. Vortex and then centrifuge the mixture at 13,000 rpm for 10 minutes at room temperature
2. Discard the liquid content using P1000 pipettor
3. Vortex and add 1ml of 1*PBS and leave at 4C for 30 minutes
4. Centrifuge at 13,000 rpm for 5 minutes
5. Discard as much liquid as possible using P1000 pipette tips
6. Add 300ul 30% Chelex in TE buffer
7. Vortex and incubate at 95C for 12 minutes. Open cap to release pressure after 2 min and vortex every 3 minutes
   - Open the caps and vortex when timer hits 10:30, 6:30, and 2:30.
8. Centrifuge at 13,000 rpm for 8 minutes
9. Transfer supernatant into clean labeled 1.5 ml centrifuge tubes
10. Centrifuge again at 13,000 rpm for 1 min
11. Transfer liquid to clean labeled 0.5 ml centrifuge tubes. Avoid Chelex particles reside at the bottom. Volume should be ~250ul for each tube
12. Put finished tubes in a white cardboard box. Good quality DNA should be of clear color, maybe light yellowish sometimes
13. Label box with a consistent labeling system and then store box at -20C

Annex 7. Protocol 2x SYBR green for plasmodium species-specific

(Snounou G, et al. 1993, Johnston SP, 2006 with modifications)

Materials and Reagents

1. Primer and probes for pf and pv
2. qPCR master mix (2 x SYBR Green qPCR master mix) Thermo Scientific
3. PCR plate (Fisher # AB1400150, $284.87/Case_150)
4. QPCR machine (QuantStudio 3, Applied Biosystems Real-Time PCR Instrument).

Reference	Species	Primer *	Sequence	Length (bp)
Snounou G, et al. 1993	*P. falciparum*	PF-F	AGTCATCTTTCGAGGTGACTTTTAGATTGCT	221
		PF-R	GCCGCAAGCTCCACGCCTGGTGGTGTC	
Johnston SP, et al. 2006	*P. vivax*	Pv-F	GAATTTTCTCTTCGGAGTTTATTCTTAGATTGC	142
		Pv-R	GCCGCAAGCTCCACGCCTGGTGGTGC	

*ThermoFisher Custom primer

qPCR reaction set up

1. Prepare the following master mix in a 1.5 mL tube (each primer at 100nM):
    a. Before starting, wrap tube in aluminum foil to protect from light.
    b. After thawing, keep qPCR Master Mix in the fridge if used frequently, store in freezer if not used for >2 weeks.
    c. Avoid frequent freeze-thaw of primers, once thawed, keep in fridge for up to a month.
2. Multiplex detection of pf and pv:

Master Mix	**1x [uL]**	**100x [uL]**
SYBR Green (2X)	10.0	1000
Pf or Pv-F	0.5	50
Pf or Pv-R	0.5	50
DNA	2.0ul	
ddH2O	7	700
Total Volume	**20µl**	**1800**

Add plasmid standards:

a. Make fresh serial dilution each day: 10^3, 10^2, 10, 5, 1 10^3, 10^2 for the qPCR, but as interim step for the lower
b. Add following concentrations 96 well plate (2 µL each):

Well	Concentration
A12	10^4
B12	10^4
C12	10
D12	10
E12	5
F12	5
G12	1
H12	1

starting from 10^4 (you will not need you need to make it concentrations) to row 12 or H in the

Seal plate with optical PCR foil

a) Use plastic tool or fingers covering with tissue to press on foil, in particular at the sides and corners
b) Centrifuge for 30 seconds (use empty plate as balance)

qPCR program running

a. Label sample wells and standard wells.
b. check the cycling conditions:
c. check the cycling conditions:

Temp	**Time**	
Hold 95°	3 min	
94°	30 sec	
55°	30 sec	
68°	1 min	45 times
Hold 95°	10 sec	

d. Confirm Total running time: ~2h and 4min.
e. In the 'Run' tab, select the instrument, press start, and save your experiment.

Annex 8. G6PD test procedure

8.1 CareSTART™ POCT S1 (using capillary or venues blood)

8.1 Specimen collection

(1) Wash hands with warm water and soap, then dry them completely. Rubbing fingers helps blood being discharged easily.

(2) Follow the lancet's instructions for use.

(3) hold the lancet (not included in the package) against the fingertip. Press the release button. When you hear a click, the puncture is complete.

(4) Discard a used lancet into safety box.

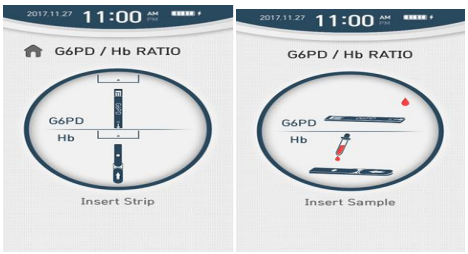
8.2 Measure G6PD/Hb Ratio.

1. Select G6PD/Hb RATIO button for starting measurement on the main menu screen.
2. Enter patient identification number using the keypad or bar code scanner and press OK button.

3. Scan G6PD and Hb QR code printed on the test strip vial. If the QR code is read successfully, the code number was displayed on the screen.

4. Make sure that the code number displayed on the screen matches the code number printed on the test strip vial, then press OK to proceed to the next step.
5. Take a new careSTART™ POCT S1 G6PD strip and Hb strip from the vial and close the lid after taking out the strip.

6. Insert the G6PD test strip into the G6PD test strip port, with the ‘G6PD’ label facing upwards. Gently push the test strip into the port until it stops. Then insert the Hb test strip into the Hb test strip port. If the strips are inserted normally, the buzzer sound and ‘Insert Sample’ screen was displayed.



7. Obtain a blood sample using the lancing device. Apply the blood sample into strips. If the sample is loaded successfully, the measurement was begin with a beep sound and the progress bar was displayed as shown below. When the progress bar reaches 100%, the measurement is completed.

8. When the measurement is completed, the result is displayed on the screen and stored in to the memory.

9. Press button to return to the main screen.