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Evaluation of diagnostic performance of multiplex real time PCR for the diagnosis of malaria in malaria elimination targeted settings of Ethiopia.

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This is to certify that the thesis prepared by Mahlet Belachew, entitled: Evaluation of diagnostic performance of molecular tests for the diagnosis of malaria in malaria elimination targeted settings of Ethiopia and submitted in partial fulfillment of the requirements for Master of Science degree in Clinical Laboratory Sciences (Diagnostic and Public Health Microbiology track) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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Abbreviations

DBS	Dried Blood Spot
EDTA	Ethylene Diamine Tetra Acetic Acid
EPHI	Ethiopian Public Health Institute
FMOH	Federal Ministry Of Health
MIS	Malaria Indicator Survey
NMSP	National Malaria Strategic Plan
PCR	Polymerase Chain Reaction
RT- PCR	Real time Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
RDT	Rapid Diagnostic Test
WHO	World Health Organization
API	Annual Parasite Incidence

Abstract

Background: Malaria incidence has declined in Ethiopia in the past ten years. Current malaria diagnostic tests, including light microscopy and antigen-detecting rapid tests (RDTs) cannot reliably detect low-density infections. Studies have shown that nucleic acid amplification tests are highly sensitive and specific in detecting malaria infection. Thus, this study took place with the aim of evaluating the performance of multiplex real time PCR for the diagnosis of malaria using patient samples collected from health facilities located at malaria elimination targeted low transmission settings in Ethiopia.

Methods: A health facility based cross sectional survey was conducted in selected malaria sentinel sites. Malaria suspected febrile outpatients referred to laboratory for malaria testing between December 2019 and March 2020 were enrolled into this study. Socio demographic information and capillary blood samples were collected from the study participants and tested at spot with RDTs. Additionally, five circles of dry blood sample (DBS) samples on Whatman filter paper and thick and thin smear were prepared for molecular testing and microscopic examination, respectively. Multiplex real time PCR assay was performed at EPHI malaria laboratory. The performance of multiplex real time PCR assay, microscopy and RDT for the diagnosis of malaria was compared and evaluated against each other.

Results: Out of 271 blood samples, multiplex real time PCR identified 69 malaria cases as *P. falciparum* infection, 16 as *P. vivax* and 3 as mixed infections. Of the total samples, light microscopy detected 33 as Pf, 18 as PV and RDT detected 43 as Pf, 17 as PV, and one mixed infection. Using light microscopy as reference test, the sensitivity and specificity of multiplex real time PCR were 100% (95% CI [93-100]) and 83.2% (95% CI [77.6-87.9]), respectively. Using multiplex real time PCR as a reference, light microscopy and RDT had sensitivity of 58% (95% CI [46.9-68.4] and 67% (95% CI [56.2-76.7]); and specificity of 100% (95% CI [98-100] and 98.9 (95% CI (96-99.9), respectively. Substantial level of agreement was reported between microscopy and multiplex real time PCR results with kappa value of 0.65.

Conclusions: Multiplex real time PCR had an advanced performance in parasite detection and species identification on febrile patients' samples than did microscopy and RDT in low malaria transmission settings. It is highly sensitive malaria diagnostic method that can be used in malaria elimination program, particularly for community based epidemiological samples. Although microscopy and RDT had reduced performance when compared to multiplex real time PCR, still

had an acceptable performance in diagnosis of malaria cases on patient samples at clinical facilities.

Key words: Malaria elimination, Multiplex real time PCR, Diagnostic performance

1. Introduction

1.1. Background

Malaria is a life-threatening disease caused by the genus *Plasmodium*. Currently, there are five species of the genus *Plasmodium* known to cause human malaria: ***Plasmodium falciparum*** (***P. falciparum)***, *P. vivax,* ***P. ovale***, ***P. malariae*** and ***P. knowlesi***. Malaria parasite is transmitted by the bite of infected female *Anopheles* mosquitoes. In *Plasmodium* lifecycle, the sporozoite stages are injected into the human host by mosquito bite, migrate via blood stream to liver and infect the hepatocytes. In the hepatocytes, the sporozoites develop into mature schizonts and release merozoites to blood, with some sporozoites remain dormant (called hypnozoites) inside the liver cells in the case of vivax and ovale infections for future malaria periodic relapse. The merozoites infect erythrocytes while certain portion of the merozoites develop into sexual forms (gametocytes) which are ingested by a blood feeding mosquito, thereby completing the lifecycle. *Plasmodium* infection causes an acute febrile illness after 7 days or longer period of incubation. The infected individual more commonly presents with clinical symptom of fever, chills, and headache. If malaria infection left untreated, it may possibly progress to severe and complicated cases which may lead to death of the patient (1,2).

Within the last decade, the incidence and malaria specific mortality rate has declined worldwide due to concerted global malaria control efforts. According to the WHO malaria report 2018, the number of malaria cases in 2017 was 43 million fewer than the cases in 2000 and 20 million fewer than the cases in 2010. Malaria burden reduced substantially in the WHO African Region since 2010 mainly due to the scaled-up of malaria control interventions, such as long-lasting insecticide-treated mosquito nets (LLINs), indoor residual spraying (IRS), and treatment of *P. falciparum* infections with artemisinin-based combination therapy and accurate diagnosis of suspected cases (3,4).

Malaria is significant public health challenge in Ethiopia since long time ago. However, in the last decade, the morbidity and mortality due to malaria infection has declined significantly. Though the first malaria eradication platform in Ethiopia was implemented in 1959 following the major malaria epidemic that hit the country in 1958, which caused 150,000 deaths, a significant decline in malaria morbidity and mortality in Ethiopia observed since 2005 after the implementation of scaling-up malaria prevention and control interventions in 2004. The last epidemic was occurred in 2003 that caused 3,000 deaths and 2 million clinical malaria cases.

Between 2004 and 2007, malaria cases and deaths in all age groups declined by 55%. A survey conducted in 2006/2007 in three regions: Amhara, Oromia, and Southern Nations Nationalities and Peoples' Region (SNNPR) showed malaria prevalence of 4.1%. According to 2007 and 2011 Ethiopian malaria indicator survey (EMIS), malaria prevalence by microscopy was around 1% in all age groups in rural malarious areas during the peak transmission season (5,6).

According to Federal ministry of health (FMoH), confirmed malaria cases in 2018 were 989,182 which is significantly lower than the cases reported in 2014 which were 2,210,298 (7). Based on this remarkable decline in malaria incidence and morbidity, the 2021-2025 national malaria strategic plan (NMSP) set a goal to achieve zero indigenous malaria in selected low transmission areas by 2025 and national elimination by 2030 (8).

Malaria transmission in Ethiopia is irregular; in most parts of the country, it is highly seasonal; but in some other parts of the country, nearly constant transmission is observed. The transmission peaks biannually, the major malaria transmission occur from September to December, following rainy season from June to August, while the minor transmission season occurs between April and May following the February to March rains. In general, malaria in Ethiopia has low and unstable transmission patterns that result in low herd immunity and significant clinical malaria illness risk after malaria infections, increased tendency for rapid progression to severe malaria, and propensity for malaria epidemics affecting all age groups of the community. *P. falciparum* and *P. vivax* are the two most dominant malaria parasites in Ethiopia which are transmitted by the bite of *Anopheline* mosquitos. *A. arabiensis* are the main vectors whereas *A. pharoensis*, *A. funestus* and *A. nili* are also widely distributed species in the country responsible for malaria transmission (9).

In the progress towards malaria elimination, the transmission has declined and the detection of infections become challenging in low transmission settings because of low parasite load (10). In order to achieve malaria elimination, it is critical to detect all infections, including those with low and sub-microscopic carriers as they constitute reservoirs in the community for significant proportion of mosquito infection and are responsible for an on-going transmission. Therefore, malaria elimination programs will have been needing highly sensitive and robust diagnostic tools for mass screening and surveillance purposes (11).

Light microscopy using Giemsa-stained blood films and RDTs are primary malaria diagnostic tools at health facilities. Light microscopy is considered as gold standard for malaria parasite

identification and confirmation at hospitals and health centres all over Ethiopia. In malaria endemic peripheral and rural areas, RDTs used as malaria diagnostic tools centred at health posts (12). RDTs are immune-chromatographic tests, identifying *Plasmodium* specific histidine reach protein-2 (HRP-2), lactate dehydrogenase protein (LDH) and aldolase. RDTs have improved sensitivity and specificity for detection of *Plasmodium* infections, require no electric source, no need of expertise to read the result and reduce time that required for performing a test, compared to microscopy. Bias during reading the result band and false negative result during hyperparasitaemia due to antigen prozone effect are limitations of RDT tests. Moreover, during mixed and low parasitaemia infection cases, both microscopy and RDTs have shown reduced performance for detection of *Plasmodium* (13). Now a day, different molecular tests are in use for the detection of *Plasmodium* species.

These molecular tests have been developed mostly based on real time quantitative PCR, with qualities of automated, quantitative and closed system that reduces the risk of contamination inherent in conventional PCR. Multiplex real time PCR has improved capability for the detection of *Plasmodium* species in low parasitaemia cases; usually it has the detection limit of less than 2 parasites per microliter and has an advantage of simultaneous detection of mixed *Plasmodium* infections, compared to microscopy and RDTs (10,14).

1.2. Statement of the Problem

Despite being preventable and treatable, malaria continues to causes significant morbidity and mortality particularly in tropics and subtropics area of the world. In 2017, due to malaria infection 219 million cases and 435,000 deaths were documented globally, 92% of these cases and deaths occur in Sub-Saharan Africa. The burden of mortality increases within high risk groups such as children under five age, pregnant woman and people living with HIV. In addition to the human losses, malaria in endemic countries is a heavy burden to the healthcare systems, while it also inhibits socioeconomic development. Consequently, intensified worldwide efforts have been exerted to prevent and control malaria disease and its burden (3).

Malaria has been the major causes for illness and death of thousands of peoples for several years in Ethiopia; this is mainly because of the country's highly variable climate as a factor of altitude and transmission patterns which favour the proliferation of malaria vector mosquito. Around 60% of the population are at risk of contracting malaria infection and 75% of the land is malarious. Plasmodium falciparum is the predominant parasite (65%) which is known to cause

the most serious infection than other species, followed by *P. vivax* (34%). The malaria transmission peaks during the harvesting season that poses a serious impact on the country's socioeconomic development (15).

Malaria morbidity and mortality in Ethiopia remarkably declining due to the implementation of scale up malaria prevention and control programme since 2005. The tremendous achievements obtained so far in reduction of malaria cases and death intensified the effort to eliminate malaria from subnational by 2020 and nationally by 2030 (11, 12). Lack of suitable tools to diagnose and treat every malaria case and surveillance tools for the evaluation of changes in prevalence, or gaps in the monitoring systems is the major challenge in national malaria elimination program(8).

The achievement of elimination of malaria require to halt every possible transmission of plasmodium parasite with in communities which demand early and accurate diagnosis followed by prompt treatment and case management of patients with malaria(16). The currently employed malaria diagnostic approaches throughout Ethiopia, microscopy and rapid diagnostic test (RDT), have poor sensitivity for detection of plasmodium species in low transmission setting that lead to underestimation of the infection prevalence. Moreover, application of malaria RDT testing in malaria control and elimination program threaten due to the emergence of *P. falciparum* with Pfhrp2 and Pfhrp3 gene deletion (17). Hence, to guide and evaluate the elimination program by getting the exact figure of the existed malaria infection, as the transmission decline, a highly sensitive malaria diagnostic tool is needed (13).

Introduction of more sensitive diagnostic tools was among strategies to be implemented by the FMOH in its endeavors to eliminate malaria from Ethiopia(18). Due to enhanced sensitivity and specificity Multiplex real time PCR assays are capable of identifying low, sub-microscopic and mixed plasmodium infection, this methods able to detect as low as 5 and fewer parasite per microliter (16). This study therefore, compared the performance of Multiplex real time PCR, microscopy and RDT for the detection of plasmodium infection from febrile patients in selected malaria Sentinel sites.

1.3. Significance of the study

Febrile illness is among the most common drivers of healthcare visits globally. In endemic countries, malaria was historically assumed to be the cause of fever. Based on 2018/19 Ethiopian NMSP report from 25 sentinel site of the total suspected cases only 25% were malaria positive with *P. falciparum* accounting for about 65%, *P. vivax* 34% and mixed around 1%(19).

In elimination targeted setting in which the parasitic carriage in individuals become dropped down, application of diagnostic tool with advanced sensitivity is critical to monitor transmission reduction and ensure elimination. The implementation of sensitive diagnostic tools into malaria elimination program is demanded in order to observe the true change in prevalence, improve the laboratory quality assessment program, and uses as a reference tool to determine limit of detection and performance evaluation of other diagnostic tools. To date different molecular techniques have been developed and shown to have superior performance over RDT and microscopy in detection of malaria parasite. Recently, different improvements have been made on these molecular techniques so as to be able to simultaneously detect, distinguish and quantify all plasmodium species within a single run. However, the performance of this method in plasmodium parasite detection was not studied well in elimination targeted settings of Ethiopia.

2. Literature review

2.1. Malaria in Ethiopia

In Ethiopia according to the 2015 malaria indicator survey (MIS) data malaria parasite prevalence were 0.5% by microscopy and 1.2% by RDT in areas <2,000 meters of altitude which are considered as malarious area and near to zero prevalence in areas above 2,000 meter (ASL) (20). Ethiopia have been developing and implementing five years NMSP since 2000 to prevent and control malaria infection, as a result dramatic achievements obtained in decreasing of malaria incidence and death. Low transmission areas increases in the past decades due to decline of malaria transmissions in areas which were considered as moderate to high malaria transmission areas as a result of scale up intervention made through NMSP (8,21).

Light microscopy and RDT are widely applicable malaria diagnostic tools. As the malaria transmission decline, the parasite carriage with in the patient blood drooped down beyond the detection limit of both methods. Lemu and his colleagues showed microscopy and RDT have low accuracy to determine the prevalence of malaria parasite using 1,435 blood sample collected from clinical and sub clinical patients, compared to PCR. In their study microscopy and RDT shown to detected 38% and 60% of the infection identified by PCR and sensitivity of these methods relative to PCR was determined to be 16.5% and 24.2% respectively. However, the sensitivity of PCR was far greater than microscopy and RDT which was 90.7% and 80%, respectively (22). In another study done in north western Ethiopia blood samples which were first recorded as *P. vivax* (7 (4.17%)), *P. falciparum* (158 (94.05%)), and mixed infections of both species (3 (1.80%)) using microscopy were re-evaluated by using SYBR green real-time PCR and samples found to be 10 (5.95%) *P. vivax,* 112 (66.67%) *P. falciparum,* 21 (12.50%) *P. falciparum* and *P. vivax* mixed infection, and 17 (10.12%) *P. ovale* positive. This revel that the performance of microscopy for species-specific and mixed infection detection is very low compared to real-time PCR (23).

2.2. Assessment of performance of malaria multiplexed qPCR

In the hospital based study in Sierra Leone, blood sample from 514 febrile patients were tested using RDT, microscopy and multiplex PCR assays parallelly. Of the total sample only 10% (65/ 514) were tested positive by the three methods. The positivity rate in the tested samples by the three methods showed great variance that, 50.2% by multiplex PCR, 24.6% by RDT, 12.8% by microscopy. This study showed the real time multiplex assay can be further modified to be

applicable in resource limited setups without compromising its superior performance (24). In a study done in a reference laboratory the multiplex qPCR assay shown to be the most analytically sensitive method (sensitivity 99.41%), followed by immuno-chromatographic tests (sensitivity of 86.47 - 88.24%) for the diagnosis of malaria when compared to reference microscopy. This study also depicted that detection limit of multiplex qPCR assay was 11 rDNA copies/µl for *P. falciparum* (25).

Application of malaria RDT testing in malaria control and elimination program threaten due to the emergence of *P. falciparum* with pfhrp2 and pfhrp3 gene deletion. Lynn Grignarda et al. developed multiplex real time quantitative PCR assay for the detection of pfhrp2 and pfhrp3 gene deletion in plasmodium falciparum in monoclonal and polyclonal infection by using pfhrp2 and pfhrp3 targeted gene and the assay shown to have 100% sensitivity and specificity in detection of pfhrp2 and pfhrp3 gene deletion and accurately estimate *P. falciparum* parasite density. In another study, a high-throughput multiplex 5' nuclease qPCR assay was developed for the detection of all 5 plasmodium species known to cause human malaria. The assay was shown clinical sensitivity and specificity of 95.8% and 98.6% for *P. falciparum*, 89.5% and 99% for *P. vivax*, 94.1% and 98.4% for *P. ovale*, 100% and 99.3% for *P. malariae* respectively and 100% specificity and sensitivity for *P. knowlesi* and 100% specificity for samples without malaria. The assay also showed improved specificity in identifying of uncertain species by microscopy into species level (26).

The performance of RDT (SD BIO-line), nested PCR and real time PCR assay in diagnosis of malaria on large scale were analyzed in one study done in Brazil, and in this study RDT shown that a sensitivity of 69.56% and specificity of 100%, while both nested and real time PCR shown similar sensitivity and specificity of 93.88% and 100% for the detection of plasmodium infection using thick blood smear as reference test respectively (27). In a study done in 2010 in Switzerland percent of agreement in species identification between multiplex real time PCR and microscopy found to be 89%(73/83), 6 of mixed infections were misdiagnosed by Giemsa-stained thin smear microscopy. This study also shown the improved capability of multiplex real time PCR assay over microscopy testing in identifying phenotypically similar species and detection of very low numbers of the co-infecting species during mixed infections (28).

In a study done to evaluate the performance of the multiplex qPCR assay in non-endemic setting, the multiplex real time PCR assay found 56 positive cases out of 839 samples, microscopy and

RDT (Pan-malarial aldolase) identified 50 and 44 of these positive samples, respectively (29). Performance of multiplex real time PCR were compared with two other conventional multiplex PCR in study done by Lau YL et.al and the sensitivity of real-time multiplex for detecting , *P. falciparum* and *P. knowlesi* infections found to be 81% when compared with other conventional PCR methods, the PlasmoNex Multiplex PCR Kit 62% and MSP-multiplex PCR 50% (30). In another study in 2004 multiplex real time PCR methods were developed for the simultaneous detection of *P. falciparum, P. vivax,* and *P. ovale* as a routine clinical diagnosis and evaluated against nested PCR as a gold standard. The result was shown to be 100% sensitive and specific for the detection and identification of these plasmodium species (31). In a study conducted at Malaysia, microscopy and nested PCR found to have sensitivity of 75% for the detection of *P. falciparum* when compared to multiplex PCR. For identification by both microscopy and nested PCR achieved 100% sensitivity compared to the multiplex PCR method. In contrast to nested PCR, multiplex PCR primers target multiple genes in a single reaction which makes it a faster and more cost effective molecular method for screening a large number of samples within a short period of time and also reduced risk of contamination (32).

3. Objectives

3.1. General Objective

- To evaluate the diagnostic performance of multiplex real time PCR for the diagnosis of malaria in malaria elimination targeted settings of Ethiopia.

3.2. Specific objectives

- To determine the performance of multiplex real time PCR and RDT in detection of malaria infection using microscopy as reference test in low malaria transmission elimination targeted settings.
- To determine the performance of microscopy and RDT in detection of malaria infection using multiplex real time PCR as reference test in low malaria transmission elimination targeted settings.
- To determine result correlation between Multiplex real time PCR and conventional diagnostic methods (RDT and Microscopy).

4. Material and methods

4.1. Study area

The study was conducted in two malaria sentinel sites namely Shoa Robit, and Metehara health centers. The health care facilities are found in malaria elimination targeted areas of Ethiopia. EPHI in collaboration with Federal Ministry of Health established 25 malaria sentinel surveillance sites, representing malarious area in Ethiopia, to control and eliminate malaria. These sites consider being eco-epidemiologically representative and focal area of malaria infection. In Ethiopia, in many parts of the country malaria transmission is seasonal, a peak malaria transmission occurs between September- December while a minor transmission season occurs in April-May, but some areas have nearly constant transmission.

4.2. The study design and period

Facility based cross sectional study was conducted by collecting data from malaria suspected febrile outpatients referred to the laboratory with in the period of December, 2019 to March, 2020 for malaria testing.

4.3. Population

4.3.1. Source population

All outpatients who were visiting the selected health center laboratories during the study period.

4.3.2. Study population

All malaria suspected febrile outpatients based on documented or self-reported history of fever with in the previous 24hrs and referred to the laboratory for malaria testing during the study period.

4.4. Inclusion and exclusion criteria

4.4.1. Inclusion criteria

All malaria suspected male and female febrile patients of any age referred to the laboratory for malaria testing were included in this study.

4.4.2. Exclusion criteria

Patients who take antimalarial therapy in the past 4 weeks before sample collection and critically ill patients were excluded from the study.

4.5. Study variables

4.5.1. Dependent variables

- Multiplex real time PCR, RDT and microscopy performance

4.5.2. Independent variables

- Age
- Sex
- Place of residence
- Clinical characteristics

4.6. Measurements and Data collection

4.6.1. Sample size calculation and Sampling technique

Sample size calculation

A total of 271 participants were enrolled in to this study. Sample size was calculated using Buderer's formula (33)

$$\frac{Z^2_{1-\alpha/2} \times S_N \times (1- S_N)}{L^2 \times \text{Prevalence}}$$

$Z_{1-\alpha/2}$ (standard normal deviate corresponding to the specified size of the critical region (α) = 1.96

SN (anticipated sensitivity) = 0.9

Prevalence= 13%

L (absolute precision desired on either side of sensitivity) = 0.1

Cause we could not find previous malaria prevalence studies done, malaria prevalence using multiplex RT PCR estimated as 13 %(intellectual guess) and the anticipated sensitivity of this method as 90% (95% [CI],80%–100%) for *P. falciparum,* compared to conventional PCR.

Sampling technique

Convenient sampling technique was used on consecutive basis to recruit the study subjects referred to the laboratory for malaria testing by attending clinical staffs according to the usual standard of care over a period of December, 2019 to March, 2020.

4.6.2. Data collection procedure

After getting patient consent, demographic profiles and clinical data were collected using structured questionnaire. Capillary blood was collected from each consented patient. Using capillary blood thick and thin smears were prepared for microscopy. RDT testing was performed and five circles of dried blood spot (DBS) was collected on Whatman filter paper and transported by maintaining cold chain to EPHI laboratory for the molecular tests.

4.6.3. Laboratory analysis

Rapid diagnostic test (RDT)

RDT testing was performed as per the manufacturer instruction using CareStart™ malaria HRP2/pLDH combo test. This test detects HRP2 and pLDH proteins specific to falciparum and plasmodium respectively. The tests were performed in the field laboratory by health center's laboratory personnel as a routine malaria testing.

Malaria microscopy

After preparation of thick and thin blood films, slides allowed to air-dried at room temperature and fixed the thin smear using absolute methanol then store at 2-8 °C until transported to EPHI. In the EPHI parasitology laboratory, slides were stained with 10% Giemsa for 10 minutes, after air dried both thick and thin smear were examined by an experienced laboratory technologist. An expert microscopist rechecked all positive slides and 10% negative slides. According to WHO malaria microscopy standard operating procedure at least 100 high power fields (HPFs) were examined for parasites detection.

DNA extraction

Genomic DNA extraction was performed using Qiagen QIAamp® 96 DNA Blood Kit(QIAGEN Inc.) from DBS sample. Briefly, three 3-mm circles of the DBS punched out and placed into a 1.5-mL tube for processing, as per manufacturer instructions and finally with 100µl volume of elution buffer the DNA was eluted and stored at -20°C until analyzed.

Multiplex real time PCR (PCR)

The PCR amplification were done by using primer and probes that targeting small unit of ribosomal RNA specific to plasmodium genus (18S rRNA) and *P. vivax* (P.v18S rRNA), and *P. falciparum* specific *var* gene acidic terminal sequence (*var*ATS). These genes have a high copy number in the malaria genome which increase the sensitivity of the PCR (34). The human RNaseP sequence is targeted as an internal control to assess the quality of DNA extraction and qPCR amplification. TaqMan fluorescence based DNA amplification and detection were performed using QuantStudio 5 Real time PCR system. For this study the real time quantitative PCR assay were run in two round during the first run all samples were tested by multiplex assay using Pan-plasmodium specific and falciparum specific primers and the second multiplex PCR run were done using *P. falciparum* and *P. vivax* specific primers. Briefly, each reaction mixture were prepared by mixing 2µl of purified DNA template, 5µl Luna Universal Probe qPCR Master Mix (New England Biolabs, Inc.), 2µl PlasQ Primer Mix and 1µl Molecular biology grade H_2O of with a final reaction mixture volume of 10µl and amplifications were carried out using thermal cycling conditions: for the first PCR run 95℃ for 1 minutes, followed by 45 cycles of 95℃ for 15 seconds and 57℃ for 45 seconds and for the second run 95℃ for 1 minutes, followed by 45 cycles of 95℃ for 15 seconds and 53℃ for 45 seconds. The 3D7 DNA standard was run in each experiment and used as a positive control and reagent control used as a negative control. For PCR run the positive control has 25 to 30 Ct value and all samples have Ct values < 30.0 for HsRNaseP taken as qualified run. Samples with Ct value between twelve to forty and sigmoidal shape amplification curve are considered as positive.

Table 1. Primers and probes sequences used for qpcr assays in this study.

Target Gene	Oligo sequence	Fluorophores	TM ◦C
Pspp18S F	GCTCTTTCTTGATTTCTTGGATG		51.71
Pspp18S R	AGCAGGTTAAGATCTCGTTCG		52.4
Pspp18S Cy5	ATGGCCGTTTTTAGTTCGTG	Cyanine 5	52
PfvarATS F	CCCATACACAACCAAYTGGA		51.78
PfvarATS R	TTCGCACATATCTCTATGTCTATCT		52.76
PfvarATS FAM	TRTTCCATAAAGGT 5'- 3'	Fluorescein	NA
Pv18S F	ACTAGGCTTTGGATGAAAGATTTTA		53.23
Pspp18S R	AACCCAAAGACTTTGATTTCTCATAA		51.65
Pv18S probe	GAATTTTCTCTTCGGAGTTTAT	Cy5-BHQ2	46
HsRNaseP F	AGATTTGGACCTGCGAGCG		53.25
HsRNaseP R	GAGCGGCTGTCTCCACAAGT		55.88
HsRNaseP_1	TTCTGACCTGAAGGCTCTGCGCG	HEX	60.62

4.7. Data quality assurance

Onsite training was given to data collectors. All blood films, DBS samples and RDT testing were performed based on standard operating procedure. The quality of each reagent was cheeked before the laboratory analysis was performed. Training on molecular testing was given to the principal investigator by senior researchers before performing the actual test. Samples and reagents were stored at appropriate temperature as indicted on the manufacturer inserts. Internal and external quality controls were run as required during analysis, all remaining samples stored at -20℃, the collected data were checked for its consistency and completeness before any

attempt to enter code and analyzed the data. Finally Epi-Info version-7 was used to control and manage errors resulting from data entry process.

4.8. Data analysis and interpretation

Data were first checked manually by principal investigator for completeness and consistency before data entry. The collected data were coded, entered into Epi-Info version-7, and was exported to STATA version 16 software (StataCorp LLC) before analysis and interpretation. Descriptive statistics was used to describe patient's socio demographic and clinical characteristics. The sensitivity, specificity, predictive values and kappa coefficient was estimated by comparing results from all three assays and 95% confidence interval was computed.

4.9. Ethical considerations

This study was approved by Institutional Ethical Review board of College of Health Sciences, Addis Ababa University and Scientific and ethical review office of EPHI(Protocol number: EPHI-IRB-219-2019). Official letter were written to sentinel sites. Written consent and assent were obtained from each study participants and the confidentiality of patient related data was maintained by avoiding possible identifiers such as name of the patient. Finally, after the whole process of data collection, all data was kept safe throughout the whole process of the research work. All participants were diagnosed and had received treatment according to the national malaria treatment guideline.

4.10. Operational definitions

Malaria case: Occurrence of malaria infection in a person in whom the presence of malaria parasites in the blood has been confirmed by a diagnostic test.

Submicroscopic infection: Low-density blood-stage malaria infections that are not detected by conventional microscopy.

Malaria suspected case: illness suspected by a health worker due to malaria, generally on the basis of the presence of fever with or without other symptoms.

Parasite density: Number of asexual parasites per unit volume of blood or per number of red blood cells.

Uncomplicated malaria: Symptomatic malaria parasitemia without signs of severity or evidence of vital organ dysfunction.

Febrile patient: Individual with self-reported fever or documented body temperature >37.5℃.

Malaria elimination: A reduction to zero of the incidence of infection caused by human malaria parasites in a defined geographical area as a result of deliberate efforts. Continued measures to prevent reestablishment of transmission are required.

5. Results

5.1. Socio demographic characteristics of study participants

A total of 271 study participants were enrolled in this study. The Mean age of the patients was 24.12 year (±14.83 SD) with 4 months minimum age and 90 years maximum age. More than half of the participants were females (54.6%) and 45.4 % of the patients were male (Table 2).

Table 2. Age and sex distribution of the study participants

Age group (year)	Female, N (%)	Male, N (%)	Total
0-5	14(5.2)	11(4.1%)	25
6-15	23(8.5)	20(7.4%)	43
16-25	72(26.6)	34(12.5%)	106
26-40	28(10.3)	44(16.2%)	72
41-64	6(2.2)	13(4.8%)	19
≥ 65	5(1.8)	1(0.4)	6
Total	148(54.6%)	123(45.4%)	271

5.2. Clinical characteristics of study participants

On the clinical presentation of study participants, among the most presented sign and symptoms, 266(98.15%) had fever, 253(93.36%) had chills, and 253(93.36%) had headache were observed. Besides sweating and muscle pain had seen in 207(76.38%) and 199 (73.43%) cases respectively. None of the patients were shown severe malaria sign symptoms (not shown on the table) (Table 3).

Table 3. Clinical characteristics of 271 study participants

Sign symptoms		Frequency	Percentage
Fever	Yes	266	98.15
	No	5	1.85
Chills	Yes	253	93.36
	No	18	6.64
Headache	Yes	253	93.36
	No	18	6.64
Sweat	Yes	207	76.38
	No	64	23.62
Muscle pain	Yes	199	73.43
	No	72	26.57
Tiredness	Yes	131	48.34
	No	140	51.66
General malaise	Yes	126	46.49
	No	145	53.51
Nausea	Yes	75	27.68
	No	196	72.32
Vomiting	Yes	58	21.40
	No	213	78.6

5.3. Laboratory results of study participants by multiplex RT PCR, microscopy and RDT

In the present study, among 271 study participants, malaria test was positive by using microscopy, RDT and multiplex real time PCR methods in 51 (19%), 61 (22.5%) and 88 (32.5%) cases , respectively. The positivity rate by three methods was increased among the age group between 16-25 years, and in male participants (Table 4).

Regarding the malaria test results in different sentinel areas, from the total 271 suspected patients, 26.2% (71) were enrolled from Shoa Robit and 73.8% (200) from Metahara sentinel site. Besides, 36.6% [26/71] of the patients were positive by multiplex PCR from Shoa Robit and 31% (62/200) of the patients from Metahara. More number of *P. vivax* (14 *vs* 2) was reported from Metahara sentinel site than Shoa Robit. Comparing the three methods, the positivity rate was highest for multiplex real time PCR 32.5% (88/271), followed by RDT 22.5% (61/271), and microscopy 18.8% (51/271), respectively. Three mixed infections (*P. falciparum* and *P. vivax*) were detected by multiplex-PCR, one by RDT and none by microscopic method. There were little differences among the three methods in detecting *P. vivax* (RDT: 17, microscopy: 18 and

multiplex real time PCR: 16). However, significant numbers of *P. falciparum* were positive by multiplex real time PCR than RDT and Light microscopy (69 *vs* 43 and 69 *vs* 33) (Table 4).

Table 4. Comparison of malaria parasite positivity by rdts, microscopy and pcr techniques by sex, age group and clinical presentation.

Characteristics		**Malaria positivity**		
		Microscopy(n=51)%	**RDT(n=61)%**	**Multiplex real time PCR (n=88)%**
Sex	Female(n=123)	15(29.4)	17(27.9)	35(40)
	Male(n=148)	36(70.6)	44(72.1)	53(60)
Age group	0-5	5(9.8)	5(8.2)	7(8)
	6-15	11(21.5)	13(21.3)	17(19)
	16-25	23(45)	29(47.5)	38(43)
	26-40	10(19.6)	11(18)	20(23)
	41-64	2(3.9)	3(5)	6(7)
	≥ 65	0	0	0
Fever	Yes (n= 266)	50	60	87
	No (n =5)	1	1	1
Chills	Yes (n=253)	50	60	85
	No (n= 18)	1	1	1
Headache	Yes (n= 253)	48	58	81
	No (n= 18)	3	3	7

Among 271 participants 266 patients were clinically presented with fever and from these febrile patients 50 were tested positive by microscopy, 60 by RDT and 87 by multiplex real time PCR for plasmodium species (Table 5).

Table 5. Frequencies of common clinical malaria sign symptoms and malaria positivity using the three malaria diagnostic methods.

		Microscopy		RDT		Multiplex real time PCR	
		Positive	Negative	Positive	Negative	Positive	Negative
Fever	Yes(n=266)	50	216	60	206	87	179
	No(n=5)	1	4	1	4	1	4
Chills	Yes(n=253)	50	203	60	193	85	168
	No(n=18)	1	17	1	17	3	15
Headache	Yes(n=253)	48	205	58	195	81	172
	No(n=18)	3	15	3	15	7	11

5.4. Diagnostic performance of multiplex real time PCR, microscopy and RDT in detecting malaria parasite

Among the total of 271 blood samples collected from febrile malaria suspected patients, 88, 61, and 51 samples were identified as positive for plasmodium infection when diagnosed by multiplex real time PCR, RDT and microscopy, respectively. Multiplex real time PCR had shown an overall sensitivity and specificity of 100% (95% CI: 93-100) and 83.2% (95% CI: 77.57-87.87), respectively and low PPV of 57.95% (95% CI: 46.95-68.4) and 100% NPV (95% CI: 98-100) compared to microscopy as reference test. However, RDT had comparable sensitivity (98.9% with 95% CI: 96.1-99.87) and better specificity (95% with 95% CI: 91.23-97.5) with microscopy than multiplex PCR. Similarly, RDT had much better PPV (81.97% with 95% CI: 70-90.6) than multiplex PCR and comparable NPV (99.52% with 95% CI: 97.38-99.9).

Using multiplex RT PCR as the reference diagnostic tool, microscopy had an overall sensitivity and specificity of 58% (95% CI: 46.95-68.4) and 100% (95% CI: 98-100), respectively. The PPV gave 100% (95% CI: 93-100) and with low NPV of 83.2% (95% CI: 77.57-87.87). The RDT had sensitivity and specificity values of 67% (95% CI: 56.2-76.7) and 98.9% (95% CI: 96.1-98.87), respectively (Table 7). The PPV and NPV for RDT was 96.7% (95% CI: 88.65-99.6) and 86.2% (95% CI: 80.77-90.55), respectively.

Using microscopy as reference test for the identification of *P. falciparum* multiplex real time PCR shown sensitivity and specificity of 100% and 83.61% and for *P. vivax*, 99.2% and 98.8% while RDT shown 100% and 95.38% for *P. falciparum* and 94.44% and 58.58% for *P. vivax*, respectively (Table 6).

Table 6. The overall* and species specific sensitivity, specificity, positive and negative predictive values of microscopy, RDT and multiplex real time PCR compared to a reference tests.

Characteristic		**Sensitivity (CI 95%)**	**Specificity (CI 95%)**	**PPV (CI 95%)**	**NPV (CI 95%)**
Real time PCR *vs* Microscopy	Overall	100(93-100)	83.2(77.6-87.9)	57.95(47-68.4)	100 (98-100)
	P. falciparum	100(89-100)	83.6(78- 88)	45.8 (34-58)	100 (98.2-100)
	P. vivax	94.4(72.7-99.9)	99.2(97.2-99.9)	89.5(66.9-98.7)	99.6(97.8-99.99)
RDT *vs* Microscopy	Overall	98.1 (89.6-9.9)	95 (91-97.5)	81.97(70-90.6)	99.5(97-99.99)
	P. falciparum	100 (89-100)	95.4(91.9-97.7)	75 (59.7-86.8)	100 (98.4-100)
	P. vivax	83.3(58.6-96.4)	98.8(96.6-99.8)	83.3(58.6-96.4)	98.8(96.6-99.8)
Microscopy *vs* Real time PCR	Overall	58 (46.95-68.4)	100 (98-100)	100 (93-100)	83.2(77.6-87.9)
	P. falciparum	45.9 (34-58)	100 (98.2-100)	100 (89-100)	83.6(78.3-88.1)
	P. vivax	89.5(66.9-8.7)	99.6(97.8-99.9)	94.4(72.7-99.9)	99.2(97.2-99.9)
RDT *vs* Real time PCR	Overall	67(56.21-76.7)	98.9 (96.-99.9)	96.7(88.7-99.6)	86.2(80.8-90.6)
	P. falciparum	56.9(44.7-68.6)	98.5(95.7-99.7)	93.2(81.3-98.6)	86.3(81.2-90.5)
	P. vivax	84.2(60.4-96.6)	99.2(97.2-99.9)	88.9(65.3-98.6)	98.8(96.6-99.8)

*** overall performance were calculated by considering all species positive as total positive by the method however, for species level performance were calculated for the detection of one species by considering other species positive by the method as negative**

5.5. Result agreement between Microscopy, RDT and multiplex real time PCR

Overall, species wise multiplex real time PCR identified 69 samples as *P. falciparum*, 16 as *P. vivax* and 3 samples as mixed infection for *P. falciparum* and *P. vivax* giving a total of 88 positive samples. From 69 *P. falciparum* samples, microscopy identified 33 samples as *P. falciparum*, 1 as *P. vivax* and 35 as negative whereas RDT identified 40 samples as *P. falciparum* 2 as *P. vivax* and the remaining 27 samples as negative. From 16 samples identified as *P. vivax* by multiplex real time PCR, microscopy detected 14 samples as *P. vivax* and 2 samples as negative. RDT identified 14 of them as *P. vivax*, 1 as *P. falciparum* and 1 sample as negative. Three samples were identified as mixed infection (*P. falciparum* and *P. vivax*) by multiplex real time PCR whereas only one sample identified as mixed infection by RDT the remaining two samples were identified 1 as *P. vivax and* 1 as negative. Microscopy detects all mixed infection as mono infection of *P. vivax* and none of the samples were detected as mixed infections by microscopy (Table 7).

Multiplex real time PCR detected plasmodium infection in all samples that were tested positive by microscopy (51 samples) and additionally 37 samples that were negative by microscopy and 59 from 61 RDT positive samples and 29 from RDT negative samples. Two samples were tested positive by RDT but negative by multiplex real time PCR. Substantial level of agreement was reported between microscopy and multiplex real time PCR with kappa value of 0.65 (% of agreement 86.35) and RDT and multiplex real time PCR with kappa value of 0.72 (% of agreement 88.56) Almost perfect agreement was reported between microscopy and RDT results (kappa value = 0.84) (Table 7).

Table 7. Percentage of agreements in identification of plasmodium spp. between microscopy, RDT and multiplex real time PCR

	Multiplex real time PCR					Percentage of Agreement	Kappa value
Microscopy	***P. falciparum***	**P. vivax**	**Mixed**	**Negative**	**Total**		
P. falciparum	33	0	0	0	33	86.35%	0.65
P. vivax	1	14	3	0	18		
Mixed	0	0	0	0	0		
Negative	35	2	0	183	220		
Total	69	16	3	183	271		
RDTs result						88.56%	0.72
P. falciparum	40	1	0	2	43		
P. vivax	2	14	1	0	17		
Mixed	0	0	1	0	1		
Negative	27	1	1	181	210		
Total	69	16	3	183	271		
RDT result	**Microscopy**						
P. falciparum	33	2	0	8	43	94.46%	0.84
P. vivax	0	14	0	3	17		
Mixed	0	1	0	0	1		
Negative	0	1	0	209	210		
Total	33	18	0	220	271		

At species level, there was little difference in percent of agreement between the three techniques in detecting *P. vivax* (real time PCR vs RDT = 98.15% with kappa value of 0.86, real time PCR *vs* microscopy = 98.89%, kappa value =0.9 and microscopy vs RDT = 97.79%, kappa value = 0.8). However, moderate agreement was reported between microscopy and real time PCR (% of agreement= 85.6, kappa value = 0.55); substantial agreement between RDT and real time PCR (% of agreement = 87.45, kappa value = 0.63); and almost perfect agreement between microscopy and RDT (% of agreement = 95.94, kappa value = 0.8) in detecting *P. falciparum* (Table 8).

Table 8. Percentage of agreements between microscopy, RDT and multiplex real time PCR at plasmodium species level.

Methods	Species	Percentage of agreement	Kappa value
Real time PCR *vs.* Microscopy	*P. falciparum*	85.61%	0.55
	P. vivax	98.89%	0.9
Real time PCR *vs.* RDT	*P. falciparum*	87.45%	0.63
	P. vivax	98.15%	0.86
Microscopy *vs.* RDT	*P. falciparum*	95.94	0.8
	P. vivax	97.79	0.8

6. Discussion

Accurate detection and early treatment of every single infection is vital to malaria elimination. Microscopy and rapid diagnostic tests have been a widely applicable malaria diagnostic tool and helping to achieve malaria control goals. However, malaria elimination requires more sensitive infection detection strategies to halt transmission (35). In this study multiplex real time PCR found to had an excellent sensitivity of 100% (95% CI: 93-100) and a good specificity of 83.2% (95% CI: 77.57-87.87) compared to microscopy as reference test. In a study done in Toronto the sensitivity of multiplex real time PCR was compared to microscopy and they reported 99.4% of sensitivity which is in line with our result. In this study, the authors showed that multiplex real time PCR is the most analytically sensitive malaria diagnostic tool (25). However, the specificity of this assay has a smaller value than microscopy (100%) and RDT (98.5%). This may be interpreted as microscopy and RDT have more false negative results compared to multiplex RT PCR test. This in turn have an implication on transmission interruption, the ultimate goal of malaria elimination program.

In this study Malaria positivity rate among malaria suspected febrile patients by multiplex real time PCR was 32.5% (88/271) which was significantly different from by microscopy (19%) and RDT (22.5%). Multiplex real time PCR detects all microscopy and 97% of RDT positive plasmodium infection. The positivity rate by all three diagnostic methods increased among younger age groups and decreased and become zero in older age group, these findings, may be associated with the fact that increased prevalence of asymptomatic infection among these group as a result of developed immunity after repeated exposure. Similarly, a study done in West Arsi Zone, Ethiopia indicated that the overall malaria positivity rate by molecular test was significantly higher than positivity rate by microscopy and RDT tests and the positivity rate among younger age group was highest when determined by microscopy, RDT and molecular test (22). Microscopy and RDT tests under estimate parasite prevalence therefore multiplex real time PCR will be an ideal malaria diagnostic tool that can be used in malaria elimination program since the program demand to observe the true change in parasite prevalence for monitoring it in accordance.

Conventional molecular tests uses multi stage procedures to detect a single parasite species at a time which is labour intensive, time consuming and prone to contamination. In spite of these limitations these molecular testes have been serving as malaria diagnostic tool for research works

and evaluation of recently developing diagnostic tools. However, Multiplex real time PCR simultaneously detects multiple plasmodium species in a single closed run. In this study all microscopy identified *P. falciparum* samples were tested positive for *P. falciparum* by multiplex real time PCR and from 18 *P. vivax* positive samples by microscopy, multiplex real time PCR identified 14 of them as *P. vivax, 1 as P. falciparum* and 3 as mixed infections. Microscopy misidentified one *P. falciparum* sample as *P. vivax* which was tested positive for *p. falciparum* by Multiplex real time PCR. This discordant result might be explained by the fact that the microscopy test quality mainly influenced by the staining quality, microscopist skill and parasitaemia. A study that conducted in southern Ethiopia was among several studies that revealed microscopy test had lower sensitivity for species identification compared to molecular tests which, cause mistreatment of the patients and lead to severe malaria, in this study 14 cases that were microscopically diagnosed as ***P. vivax*** were found positives for ***P. falciparum*** when retested by nested PCR (36).

PCR has been considered as a molecular tool for Plasmodium detection and species identification. In addition to the detection of low parasitemia and speciation, studies have shown that this technique is robust in identifying mixed species infection that are often undetected and under reported by RDT and microscopy assays. Detecting of mixed infections provides accurate information for patients' treatment and epidemiological studies regarding malaria transmission. (23,37,38). In the present study, among three mixed infection identified by PCR, RDT detected only one and microscopy none. RDT identified the rest two as *P. vivax* and negative and microscopy all three mixed samples as *P. vivax*. This may be due to very low level of parasitaemia of the co-infecting species during mixed infections. likewise in a study done in Israel, from ten mixed infections identified by real-time PCR, only one was identified by microscopy and RDT testing(39). In another study conducted in Switzerland to evaluate microscopy and multiplex real time PCR correlation, multiplex qPCR assay correctly identified to the species level and mixed infections with low levels of parasitaemia but 71% of mixed infections were misdiagnosed by microscopy (28). In a study done to determine the prevalence of mixed infection using real time PCR in northern Ethiopia the prevalence of mixed infection was 1.8% by microscopy and 12.5% by real time PCR from a total of 168 samples (23). As proved by results from these studies, multiplex real time PCR has the most notable advantages of higher sensitivity to detect mixed infections and to identify species of malaria parasites

accurately. Therefore, it is the most convenient malaria diagnostic methods for countries like Ethiopia, where *P. falciparum* and *P. vivax* are co-endemic species, unlike most African countries where *P. vivax* has low or nil endemicity (5).

The RDT test and microscopy had shown lower performance compared to multiplex qPCR in our study. However results from both assay shown almost perfect agreement. Two samples were positive for *P. falciparum* by RDT and tested negative by both PCR and microscopy. These two false positive results might be due to the perseverance of HRP2 antigen in the blood after treatment or cross-reaction of antibody that coated on RDT with a rheumatoid factor (40). Furthermore, one sample was tested negative by RDT and it was *P. vivax* positive by both PCR and microscopy this false negative result might be associated with limitations of pLDH-based tests, and these tests had decreased sensitivity at low parasitaemia and performance of detection highly affected by the storage and transportation conditions than HRP2 based tests (41). Another explanation might be the prozone effect of hyper parasitaemia which lead to false negative result in RDT testing (10). In a study of comparative analysis between molecular and serological diagnosis of malaria on large scale done in Brazil indicated that the sensitivity and specificity of RDT test when compared to multiplex qPCR were found to be 69.56% and 100%, respectively, which was concordance with our study result (sensitivity of 67% and specificity of 98.9%) (27). There was another study which evaluates the performance of qPCR and RDTs for the diagnosis of malaria in returning traveller from endemic area and qPCR found to have comparable sensitivity (99.41%) and greater specificity (90.88%) than the current study, compared to microscopy as reference test. However, in our study the sensitivity of carestart RDT, compared to microscopy as reference test found to be much better than the sensitivity of carestart RDT, found in this study (25).

Though, identifying malaria into species level have crucial impact on patient managements and transmission interruption, RDT testing are incapable to differentiate and have decreased performance in detecting non-falciparum malaria. In our study the sensitivity of RDT for detecting *P. vivax* were 83.33 % and for *P. falciparum* were 100% compared to microscopy as reference test. This result was higher than the sensitivity for *P. falciparum* of similar RDT format in a study done by Feleke et al.(94.4%) and Moges et al.(92.9%) but lower sensitivity for *P. vivax* than both study (85% and 90.9%, respectively) (42,43).

In this study, nearly all (87/88) multiplex real time PCR positive cases were from patients presented with self-reported fever which was large proportion when compared to febrile cases identified by RDT and microscopy. The negative predictive value of multiplex real time PCR found to be 100% (95% CI: 98-100) using microscopy as a reference test. Diagnostic tool with high negative predictive value reduce the probability of missing infection in individuals, means when the patient is malaria negative using multiplex real time PCR, plasmodium infection could be ruled out with high certainty. This quality of PCR makes it an ideal diagnostic tool to be used in malaria elimination endeavors than RDT and microscopy which were found to have high positive predictive value and low negative predictive value in current study. RDT and microscopy malaria diagnosing increase the probability of missing plasmodium infection which have negative impact on transmission interruption since all infected individuals considered as potentially infectious and thus potentially able to contribute to transmission(44) .

Multiplex real time PCR identified 69 malaria cases as *P. falciparum* infection, 16 as vivax and 3 as mixed infections from the total of 271 symptomatic malaria suspected patients in this study. Of these malaria cases large number of *P. falciparum* cases missed by both RDT and microscopy testing however, all three methods showed perfect agreement in *P. vivax* species identification. Additionally, the specificity of multiplex real time PCR for *P. vivax* identification (98.8%) were much better than *P. falciparum* identification (83.61%) when compared to microscopy as reference test. This is probably due to that the presence of all erythrocytic stage of *P. vivax* in peripheral bloods that increase parasitic densities in the blood of symptomatic patients but *P. falciparum* causes cytoadherence and sequestration of infected erythrocytes that reduced parasite densities from peripheral blood(45). Using microscopy as reference test, multiplex real time PCR showed excellent sensitivity for both *P. falciparum* (100%) and *P. vivax* (99.2%) species identification in this study. Which is closely related to the finding of study done at Bangladesh on clinically suspected malaria patients that the sensitivity of real time PCR for *P. falciparum* and *P. vivax* identification using microscopy as a gold standard were 97.1% and 95.2% respectively (46).

Substantial numbers of *P. falciparum* infection was detected by multiplex real time PCR than microscopy and RDT methods. This might be explained as follows, in this study varATS TaqMan PCR method were used for *P. falciparum* detection which is a highly sensitive PCR primers for malaria than 18S rRNA based PCR which were also used in a current study for the

detection of *P. vivax* (34). Another reason may be due to the biology of the parasite in which the parasite has a tendency to sequestrated in the organs during its life cycle and unable to detect by RDTs and microscopy.

7. Limitations of the study

In this study multiplex real time PCR assay was performed only for two plasmodium species. Although the two plasmodium species are predominantly prevalent in Ethiopia, recent studies have shown that other species also have been seen in the country. Due to reagent shortage limit of detection (LOD) of multiplex real time PCR were not evaluated. Moreover, the multiplex real time PCR was only evaluated against conventional malaria diagnostic methods. It could be better to include other PCR methods such as conventional PCR for evaluation.

8. Conclusion

Multiplex real time PCR was the most sensitive malaria diagnostic method that can be used in malaria elimination program. It had an advanced performance in species identification and mixed infection detection than microscopy and RDT in low malaria transmission settings and showed better performance in detection of plasmodium infection among febrile patients. Therefore, this assay can be used for epidemiological and community based prevalence studies and for verification of elimination in areas where malaria elimination was launched. However, microscopy and RDT still have an acceptable performance to be used as a malaria diagnostic tool in health facilities for patients' treatment due to the fact that they are affordable and easily performed diagnostic methods.

9. Recommendation

Generally, multiplex real time PCR has an excellent diagnostic performance in low malaria transmission settings. Therefore, this assay should be considered in malaria diagnostic methods in Ethiopian NMSP. However, further study needed

- to determine LOD of this method,
- evaluate its performance for the detection of the remaining plasmodium species other than falciparum and vivax,
- cost analysis and further evaluation using another PCR method as a gold standard

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11. Annex

Annex-I Information sheet and consents form

A. Information sheet (English version)

Title of the Research Project: Evaluation of diagnostic performance of molecular tests for the diagnosis of malaria at EPHI, Addis Ababa, Ethiopia.

Principal Investigator: Mahlet Belachew (BSc, MSc candidate)

Name of the Organization: Ethiopian public health Institute; Addis Ababa University College of Health Sciences Department of Medical Laboratory Sciences.

Purpose of the Research Project

We are asking you to take part in this study as a study subject of our endeavour to evaluate the malaria diagnostic methods. Findings of the research can be an input for the national malaria elimination program to help improve the laboratory diagnostic methods for early and accurate detection of the disease. Therefore, at the end of the study based on the result found all the necessary recommendations will be forwarded to all responsible bodies.

Procedures and the expected participation

If you are willing to participate, you need to sign a consent form and respond to a short questionnaire interview. Not only this but also blood specimen will be collected from you. The required blood sample will be collected by medical laboratory staff by finger pricking.

Potential risks

During collection of specimen from you finger pricking may cause you a little bit of pain. However, appropriate precaution will be taken and all samples will be collected by trained health professionals. If anything happened, appropriate medical care will be provided to you.

Confidentiality

All information you give and data obtained from laboratory analysis will be kept confidential and will be communicated only to responsible figures. Formats containing data will be kept locked.

Benefits

There will be no immediate benefits in your participation in the study; you will not receive any payment for your participation in this research study as compensation. However, when the study results are known it will provide information about malaria diagnostic methods to malaria elimination program and helps to achieve malaria elimination which will benefits you as a community

Participation and Withdrawal from the Study

The participation is voluntary and you have the right not to participate in this study. You may withdraw at any time and place without consequences of any kind. You may also reject to give any sample. You can ask any questions regarding this study and you have a right to get a laboratory diagnosis result free.

Contact information

If you have any questions about this study you can contact the following principal investigators and advisors for further information.

Mahlet Belachew **Phone:** 0913361767 **E-mail:** mahibel25@gmail.com

Mr. Adugna Abera **Phone**: 0911 883 483

Scientific and ethical review office of Ethiopian Public health institute Tel: 0118685503/ (15)

B. Consent form for adults (≥18 years)

Code No. _____

I have read the information above, or it has been read to me. I have been given the opportunity to ask questions and my questions have been answered to my satisfaction. **I voluntarily consent that I would participate in this study.** To collect my blood and be a participant in this study, I understand that I have the right to withdraw from the study at any time.

Print name of participant, date and signature or thumb impression of participant

_____ ____ /____ /____ (dd/mm/yy) _____

If illiterate;

Print name of independent literate witness, date and signature of witness (if possible, this person should be selected by the participant and should have no connection to the research team)

_____ ____/____/_____ (dd/mm/yy) _____

Phone number_____

Print name of researcher, date and signature of researcher

_____ _____/____/_____ (dd/mm/yy) _____

C. Consent form for adults (≥18 years) (Oromiffa version)

Lakk. _____

Kaayyoon, faayidaan, akkataa fi icitiin qorannoo kanaa maal akka ta'e naaf dubbifamee ykn dubbisee naaf galeera. Qorannoo kana yoon hin barbaadne ta'e yeroo kamiyyu gadhisee keessaa bahuuf mirgaa guutuu akkan qabus hubadheera. Anis qorannoo kana keessatti qooda fudhachuuf hayyamamoo ta'uu koo, qoranichaaf ragaa barabaachisaa ta'ee dhiiga, akkasumas odeeffannoo kennuu akka ta'ee fi faayidaan ani argadhuus bu'aan qorannoo kanaa kaffaltii tokko malee bilisaan akka naaf adeemsifamu hubadheera.

Ani_____ odeeffannoo fi dhiiga qorannoo kanaaf barbaachisu kennuuf walii galuukoo nan mirkaneessa.

Mallattoo_____Guyyaa_____/_____/_____(guyyaa /ji'a / bara)

Annex-II Questionnaire

This questionnaire records both socio-demographic and clinical characteristics. For each question please give the answer carefully. Your name is not included in the questionnaire and they are completely anonymous and confidential. Your answers will be kept only by the study investigators and will not be distributed to anyone else.

Questionnaire no: …….

Name of interviewer: …….

Date: …….

Part 1 – Socio demographic characteristics

1.1. ID no. of suspect …….

1.2. Age …….

1.3. Sex …….

1.4. Permanent address: City ……. Woreda ……. Keble…….

1.5. How long have you been staying in this area (if permanent address is different)?

1.6. Have you ever been travel outside this area with in the last three months? And where was the place?

Part 2 Clinical characteristics

2.2. Does the patient currently have any of the following symptoms?

Fevers	□ Yes	□ No
Chills	□ Yes	□ No
Sweats	□ Yes	□ No
Headache	□ Yes	□ No
Muscle pains	□ Yes	□ No
Nausea	□ Yes	□ No
Vomiting	□ Yes	□ No
General malaise	□ Yes	□ No

2.3. Does the patient have any of the following signs?

A temperature greater than 37.5℃	□ Yes	□ No
Perspiration	□ Yes	□ No
Tiredness	□ Yes	□ No

2.4. Does the patient have any of the following features of severe malaria?

Yellow discoloration (jaundiced) □ Yes □ No

Rapid breathing □ Yes □ No

Impaired consciousness □ Yes □ No

2.5. Did you ever diagnose with malaria? □ Yes □ No

2.6. Have you taken any anti-malarial medication in the last four weeks? □Yes □ No

Annex-III Laboratory data recording formats

Sample collected for: Microscopy □

RDT □

DBS □

Id No	RDT result				Microscopy Result				
	Negative	Positive			Negative	Positive			
		P. f	P. v	Mixed		P. f	P. v	Mixed	Parasitic density

Annex-IV Laboratory procedure

A. Manual DNA Extraction Protocol for Dried Blood Spot Filter papers

(Modified for use with QIAGEN QIAamp® 96 DNA Blood Kit Cat #: 51161 OR 51162)

1.0 Purpose

This (modified) protocol provides a manual method for extracting DNA from dried blood spots on filter paper when used in conjunction with Qiagen QIAamp® 96 DNA Blood Kit.

2.0 References

2.1 Handbook for Qiagen QIAamp® 96 DNA Blood kit

3.0 Materials

3.1 Qiagen QIAamp® 96 DNA Blood Kit (Qiagen; cat# 51161 or 51162)

3.2 Buffer ATL (Qiagen cat# 19076)

3.3 Ethanol (96 – 100%)

3.4 Scissors and forceps (for cutting strips of filter paper with dried blood spots)

3.5 70% alcohol (Ethanol or Isopropanol)

3.6 S-Blocks (Qiagen cat# 19585, if purchased separately)

4.0 Equipment:

4.1 56°C incubator

4.2 Table - top centrifuge (Eppendorf 5810R with Eppendorf A-2-DWP rotor) (recommended for use with Qiagen S-blocks)

4.3 Calibrated 8 or 12 well multichannel pipets (2-20 µL and 20-200 µL) (Matrix or comparable) and aerosol resistant pipette tips

4.4 Calibrated Micropipets (10 µL, 20 µL & 1000 µL) (Matrix EDP Plus or comparable) and aerosol resistant pipette tips

4.5 Reagent reservoirs (for multichannel pipettes)

4.6 Freezer (-80°C, for long tern storage of DNA)

5. Safety and Precautions

5.1 Use proper lab etiquette when working in the laboratory. Wear gloves, labcoat and safety glasses when handling specimens.

5.2 Use appropriate safety precautions when using razor blade or scissors to cut filter strips with dried blood. Wipe the cutting tool with 70% alcohol between samples.

5.3 Ensure that all equipment (pipettes, centrifuge, water bath, etc.) is properly calibrated prior to use.

5.4 Take utmost care during pipetting steps to prevent cross contamination of samples.

5.5 Store extracted or amplified DNA at -80 °C (2-8 °C storage is allowed for ≤ 24 hours; -20 °C storage is allowed for ≤ 2 weeks).

5.6 Check the bottles of ATL and AL buffers. If a precipitate has formed, dissolve by incubating at 56 °C before use.

6.0 Procedure

6.1 Make sure incubator is at 56°C.

6.2 Resuspend the lyophilized Qiagen Protease stock solution in the protease solvent provided with the kit.

Note: Dissolved Qiagen Protease is stable for up to 2 months when stored at 2-8°C. Alternatively, it can be aliquoted into single use aliquots and stored at -15°C to -30°C for prolonged storage.

6.4 Before using for the first time, add 125 mL ethanol (96-100%) to the bottle containing Buffer AW1. Mix well. Write date of reconstitution and store at room temperature.

6.5 Before using for the first time, add 160 mL ethanol (96-100%) to the bottle containing Buffer AW2. Mix well. Write date of reconstitution and store at room temperature.

6.6 Using clean 3mm DBS puncher transfer 3 circles of filter paper strips stained with dried blood into the bottom of the collection microtubes (included in the kit).

6.8 Prepare ATL/Proteinase K solution:

For each extraction, take 180 µL of Buffer ATL. Add 20 µL reconstituted Protease. Mix well. (Make extra volume to allow for loss during pipetting, use for negative control, etc).

6.8.1 No. of filter samples extracted = _____ x 0.02 mL Protease + 0.1 mL (extra) = _____ mL Protease.

6.8.2 No. of filter samples extracted = _____ x 0.18 mL Buffer ATL + 0.9 mL (extra)
= _____ mL Buffer ATL

6.8.3 Mix the volumes of Protease and ATL buffer calculated in steps 6.8.1 and 6.8.2.

6.9 Add 200 µL Protease/Buffer ATL solution to each collection microtube. Seal the microtubes tubes using collection microtube caps provided.

6.10 Incubate at 56 °C overnight with shaking.

6.11 Next day, briefly centrifuge to remove any solution from the caps.

6.12 Add 200 µL of Buffer AL to the sample, mix by thoroughly shaking for 15 seconds (hold collection microtubes and shake up and down). Centrifuge briefly at 1811 rcf to collect any solution from the caps and incubate at 56 °C for 15 min.

6.13 Add 200 µL ethanol (96 – 100%) to each well. Seal the wells with new caps & shake vigorously for 15 seconds. Centrifuge briefly at 1811 rcf to collect any solution from the caps.

6.14 Place a QIAamp 96 plate on top of an S-Block (both provided with kit).

6.15 Carefully apply the mixture from step 6.13 (approx. 600 µL per tube) from the collection tube to the QIAMP 96 plate. (Take care not to wet the rims of the wells to avoid aerosol formation)

6.16 Seal the QIAamp 96 plate with an AirPore tape sheet (provided). Centrifuge the QIAMP 96 plate including the S-block at 2130 rcf for 8 min.

6.17 Remove the AirPore tape sheet. Carefully add 500uL of buffer AW1 to each well. Empty S-block and replace QIAamp 96 plate onto S-block. Seal the QIAamp 96 plate with a new AirPore tape sheet.

6.18 Centrifuge at 2130 rcf for 5 min.

6.19 Remove the tape. Carefully add 500 µL of buffer AW2 to each well. Empty S-block and replace QIAamp 96 plate onto S-block. **Do not seal with AirPore Tape** to allow for sufficient ethanol evaporation.

6.20 Centrifuge at 2130 rcf for 25 min.

6.21 Place the QIAamp 96 plate on top of a rack of elution microtubes (provided with kit).

6.22 To elute DNA, add 150 µL Buffer AE to each well using a multichannel pipette. Seal the QIAamp 96 plate with an AirPore tape sheet and incubate for 1 minute at room temperature.

6.23 Centrifuge at 2130 rcf for 8 min. Seal the elution plate with caps (provided with kit) and store extracted DNA (eluate) at -80 °C.

B. **Standard Operating procedure (SOP) for Malaria qPCR Assay**

Abbreviation and terms

SOP - Standard Operating Procedure
qPCR - quantitative Polymerase Chain Reaction
PlasQ assay - multiplex qPCR assay to quantify *P. falciparum*
Pf - *Plasmodium falciparum*
Hs - *Homo sapiens*
Pspp - *Plasmodium spp*
NTC - Non-template Control
PC - Positive Control
RT - Room Temperature
FAM - Fluorescein fluorophore
YYE - Yakima Yellow fluorophore
Cy5 - Cyanine 5 fluorophore
Ct - cycle threshold
DNA -Deoxyribonucleic acid

Introduction

The qPCR assay (herein referred to as PlasQ assay) described in this SOP consists of two independent Plasmodium targets combined in a multiplex assay. The *Pan-Plasmodium* 18S

rRNA sequence (Pspp18S), and the *Plasmodium falciparum* specific var gene acidic terminal sequence (PfvarATS) are targeted. The human RNaseP sequence is targeted as an internal control to assess the quality of DNA extraction and qPCR amplification. This strategy of using two *Plasmodium* targets ensures high specificity and increased sensitivity of *Plasmodium falciparum* and *non-falciparum* species detection during Controlled Human Malaria Infection studies as well as epidemiological studies.

Equipment, materials and reagents

Equipment should be maintained and calibrated according to the manufacturer's instructions or in-house guidelines. Equipment records should be kept and updated as appropriate.

Instrument

- PCR machine (FAM, VIC and CY5 channel are required)
- Vortex -
- Centrifuge -
- Pipettes including sterile, filtered tips (Volume range: 0.5 – 1000 µL)
- Refrigerator
- Freezers
- Powder free latex gloves

Consumables

- 96 well qPCR plates (Sterile and DNase-free)
- PET transparent plate seals
- 1.5 mL Eppendorf tubes (Sterile and DNase-free)
- 2 mL Cryovials, sterile

Reagent and storage

- H_2O for Molecular Biology, DNase-free RT
- Luna Universal Probe qPCR Master Mix -20 °C
- Standard for *P. falciparum* DNA Nucleic Acid Amplification Techniques -20 °C
- Primer and Probes:

Name	Sequence	Oligo modification
Pspp18S F	GCT CTT TCT TGA TTT CTT GGA TG	-
Pspp18S R	AGC AGG TTA AGA TCT CG TTC G	-
Pspp18S Cy5	ATG GCC GTT TTT AGT TCG TG	Cy5-BHQ2
HsRNaseP F	AGA TTT GGA CCT GCG AGC G	-
HsRNaseP R	GAG CGG CTG TCT CCA CAA GT	-
HsRNaseP YYE	TTC TGA CCT GAA GGC TCT GCG CG	YYE-BHQ1
PfvarATS F	CCCATACACAACCAAYTGGA	-
PfvarATS R	TTCGCACATATCTCTATGTCTATCT	-
PfvarATS FAM	TRTTCCATAAATGGT	FAM- NFQ/MGB

Procedure

Primer Mix preparation

A 5x PlasQ Primer Mix is prepared using the 100 µM stock solutions of all primer and probes. The 5x Primer Mix can be kept for up to three months at – 20 °C.

To prepare 1 mL of 5x PlasQ Primer Mix following volumes are pipetted into a 1.5 mL Eppendorf tube:

Primer	Stock concentration [µM]	Final concentration in 5x Primer Mix	Volume in µL
Pspp18S F	100	2	20
Pspp18S R	100	2	20
Pspp18S Cy5	100	1	10
PfvarATS F	100	1	10
PfvarATS R	100	1	10
Pf varATS FAM	100	0.5	5
HsRNaseP F	100	1	10
HsRNaseP R	100	1	10
HsRNaseP YYE	100	0.5	5
Molecular biology grade H_2O			900

Master Mix preparation

Determine the number of samples to be analyses plus controls and 10% overhang that will be run simultaneously on one qPCR amplification plate. Multiply this number of samples by the following volumes:

Component	**Stock concentration**	**Final Concentration**	**Reaction volume**	**Example for 100 reactions**
Luna Universal Probe qPCR Master Mix	2x	1x	5µL	500µL
PlasQ Primer Mix	5x	1	2µL	200µL
Molecular biology grade H_2O	-	-	1µL	100µL

- Pipette all qPCR Master Mix components into a 1.5 mL Eppendorf tube
- Gently mix the Master Mix by vortexing.
- Transfer 8µL of the qPCR Master Mix to each well
- Add 2µL Molecular biology grade H_2O to the NTC samples
- Transfer the qPCR plate to the DNA addition bench.
- Add 2µL template DNA to each well or tube
- Add 2µL of PC
- Add 2µL of NC
- The FINAL reaction volume including template DNA is 10µL.
- Briefly centrifuge the plate to spin down the content.

Start the qPCR run:

- Place the plate or tubes in the instrument.
- Select the PlasQ qPCR run conditions which are as follows:

Step	Temperature	Time	Cycles
Polymerase activation	95 °C	1 min	
Denaturation	95 °C	15 sec	45x
Annealing and Elongation	57 °C	45 sec	

- Select the PlasQ qPCR plate design
- Enter the sample IDs and start the run.

Result interpretation

- Amplification curves have a typical qPCR-like sigmoidal shape. Exclude the wells with non-sigmoidal curves from analysis.
- There is no amplification in NTC. If there is amplification in NTC repeat the run.
- PC has a Ct value 25.0-30.0 for Pspp18S and PfvarATS. If Ct value > 30.0 repeat the run.
- All samples have Ct values < 30.0 for HsRNaseP. If Ct value for HsRNaseP > 28.0 repeat DNA extraction.

Declaration

I, the undersigned agree to accept responsibility for the scientific ethical and technical conduct of the research project and for provision of required progress reports as per terms and conditions of the research publications office.

M.Sc. candidate: **Mahlet Belachew (B.Sc.)**

Signature: _____

Date of submission: _____

This thesis has been submitted with our approval as advisors.

Advisor: **Mistire Wolde (MSc, PhD)**

Signature: _____

Date: _____

Place: Addis Ababa, Ethiopia.

Advisor: **Adugna Abera (MSc)**

Signature: _____

Date: _____

Place: Addis Ababa, Ethiopia.