EVALUATION OF NXTEK™ ELIMINATE MALARIA *P.FAG*AGAINEST qPCR TO DETECT ASYMPTOMATIC *PLASMODIUM FALCIPARUM* MALARIA AMONG SCHOOLCHILDREN IN JIMMA ZONE; GOMMA DISTRICT, ETHIOPIA

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

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EVALUATION OF NXTEK^{TM} ELIMINATE MALARIA *P.FAG* AGAINEST qPCR TO DETECT ASYMPTOMATIC *PLASMODIUM FALCIPARUM* MALARIA AMONG SCHOOLCHILDREN IN JIMMA ZONE; GOMMA DISTRICT, ETHIOPIA

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ABSTRACT

Background: *Plasmodium falciparum* (*P. falciparum*) histidine-rich protein2 (HRP2) and pan-Plasmodium lactate dehydrogenase (pLDH)-based rapid malaria diagnostic tests (RDTs) play a critical role in malaria control in endemic countries. *NxTek*^{TM} *Eliminate Malaria PfAg*is newly developed HRP2-based highly sensitive malaria RDT. However, hardly any data on its field diagnostic performances are not available in the Jimma zone in south-western Ethiopia.

Objective: To evaluate the diagnostic performance of the *NxTek*^{TM} *Eliminate Malaria PfAg*against quantitative PCR (qPCR) for the detection of asymptomatic *P. falciparum* malaria infection amongschoolchildren from September –December2021.

Methods:A school-based cross-sectional study was conducted from September to December 2021on 994 healthy school children (aged 5 to 15 years) using the multi-stage sampling technique. Finger-pricked blood samples were collected for microscopy, *NxTek*^{TM} *Eliminate Malaria PfAg*, conventional RDT (Co-RDT), SD Bioline, and QuantStudio Multiplex PCR. Giemsa-stained blood smears were examined microscopically. Dry blood spots (DBS) were collected for qPCR assay.

Results: The overall prevalence of asymptomatic malaria was 5%. The prevalence of *P. falciparum* was 4.5% by qPCR, 2.2% by *NxTek*^{TM} *Eliminate Malaria PfAg*/Co-RDT, and 1.5% by microscope. Compared to qPCR, the sensitivity of *NxTek*^{TM} *Eliminate Malaria PfAg*was 44% and the specificity 100%. PPV and NPV were 100% and 97.43%, respectively. The accuracy of the test was 97.5%. The kappa value was reported as 0.62, indicating substantial agreement between the methods. Compared to the microscope, the sensitivity of the *NxTek*^{TM} *Eliminate Malaria PfAg*was 68.2% while the specificity was 100%. PPV and NPV were 100% and 99.5%, respectively. The accuracy of the test was 99.5%. The kappa value of 0.81 shows perfect agreement between the test methods.

Conclusion: The results presented here suggest that *NxTek*^{TM} *Eliminate Malaria PfAg*has a satisfactory agreement with qPCR and is a more sensitive tool than the microscope in asymptomatic children and could represent an alternative diagnostic tool for malariacontrol programs.

Keywords: *NxTek™ Eliminate Malaria P.f Test*, asymptomatic malaria, school-children, Jimma, Gomma district.

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LIST OF ABBREVIATIONS

API……Annual Parasite Index

CC…….Curative Care

CI…….. Confidence Interval

DDT…….. Dichloro diphenyl trichloroethane

EPSA……. Ethiopian Pharmaceutical Supply Agency

FMoH……...Federal Ministry of Health

HPF……High Power Field

ICEMR…….International Centers of Excellence for Malaria Research

IRB……Institutional Review Board

IRS……Indore Residual Spray

ITNs……...Insecticide Treated Nets

mRDT ……...malaria RDT

NMCPs …… National Malaria Control Programs

NPV……...Negative predictive value

Pf……Plasmodium falciparum

Pfhrp-2……Plasmodium falciparum histidine-rich protein-2

PfLDH……Plasmodium falciparum lactate dehydrogenase

PPV……Positive predictive value

PSC…… Pre-School Consultation

PV……...Plasmodium Vivax

PVPLDH……...Plasmodium vivax specific Lactate Dehydrogenase

RBCs…….Red Blood cells

RDT……...Rapid Diagnostic test

SOP……...Standard Operating Procedures

SPSS…….Statistical Package for Social Science

USFDA……..The United States food and drug administration

WBCs……White Blood Cells

WHO…….World Health Organization

CHAPTER ONE: INTRODUCTION

1.1. Background

Malaria is a serious parasitic disease caused by a bite of infected female *Anopheles* mosquitoes, resulting in the transmission of protozoan parasites to humans. It is a major public health problem in endemic countries, resulting in loss of life every year, particularly among mothers and children's(1). According to the WHO report 2021, the estimated number of malaria cases increased to 241 million cases, compared to 2019 an additional 14 million cases are reported. In Ethiopia, malaria is one of the main killers and 68% of the population is living in the malarious area where *P. falciparum* accounted for 60% while *P. vivax* 40%(2).However, the 2021 World Malaria Report shows alarming results. In 2019 there were 904,495 positive cases confirmed by the microscope, while in 2020 1,743,755 cases were reported. This means 839,260 more malaria cases in 2020 than in 2019. Of the positive cases, 1,340,869 (76.9%) were *P. falciparum* and 263,877 (15.1%) *P. vivax*cases. Mixed and other infections account for the remainder (30,051 and 108,958). During the same period, there were no WHO reports on malaria RDT positivity rates(1). This report shows that there could be a disruption in malaria intervention strategies or a resurgence of malaria cases in newor previous areas in the country.

One of the critical roles of malaria diagnostic tools is a rapid and accurate diagnosis for effective malaria management(3). The global impact of malaria has encouraged the industries to develop convincing diagnostic tools/strategies not only in developed countries but also in resource-constrained settings where expertise in the field is lacking(4).Therefore, the aim of the new diagnostic innovations is to avoid unnecessary costs for malaria treatment, prevent drug resistance, and improve case reports(5).These diagnostic strategies include Malaria Rapid Diagnostic Tests (mRDT), which have transformed malaria diagnosis and were recommended by the World Health Organization (WHO) in 2010(6). Which are designed as rapid Immunochromatographic tests used to identify specific antigens from malaria parasites in the blood such as *P. falciparum*HRP2 and pLDH. PfHRP-2 is specific for the diagnosis of *P. falciparum*, while PfPLDH and *P. vivax*-specific lactate dehydrogenase (PVPLDH)-based RDT are specific for the diagnosis of *P. falciparum* and *P. vivax,* respectively. On the other hand, pan-specific PLDH-based RDT detects the presence of all human malaria species(7).

Global sales of RDTs from 2010 to 2020 were 3.1 billion, of which more than 81% are estimated to have been distributed to sub-Saharan Africa. Of the total RDTs distributed worldwide by the National Malaria Programs (NMP), an estimated 64% were *P. falciparum*only detected and shipped to sub-Saharan Africa(8, 9).In malaria-endemic settings, RDTs are used as analternate for microscopic diagnosis and have demonstrated approximately 100% sensitivity for detection of *P. falciparum*at densities greater than 100 asexual parasites/ μL or > 0.002% parasitized red blood cells(10).

Usually, light microscopy is the gold standard for detecting malaria parasites. It can detect malaria parasites even at low densities of five to ten parasites/μL of blood when used by experienced technicians(11, 12).However, microscopic examination to diagnose malaria is labor-intensive and time-consuming. In a clinical setting, the microscopic technique is routinely used as a diagnostic reference method, while at the community level, where microscopy is not affordable, RDTs are commonly used to study, treat, and monitor malaria cases in malaria-endemic areas, including Ethiopia(13).

It has been suggested that the performance of RDTs in different malaria-endemic areas needs to be continuously monitored and evaluated. For the success of the elimination program, quality-assured malaria RDTs are routinely used in malaria-endemic countries(14). Recently, *NxTek*^{TM} *Eliminate Malaria Pf Ag*with product codes 05FK140, 05FK141, 05FK142, and 05FK143 (Abbott Diagnostics Republic of Korea Inc., CE marked regulatory version) has been included in the WHO Prequalified *In- vitro* diagnostics and was listed on April 12, 2019(15). It was developed in response to the diagnostic challenges of detecting low-density *P. falciparum*parasitemia associated with asymptomatic malaria infections as a point-of-care diagnosis(16)**.**Several attempts have been made to evaluate the diagnostic performance of available RDTs using blood smear microscopy and polymerase chain reaction (PCR) used to verify positive and conflicting results(17).

Although the *NxTek*^{TM} *Eliminate Malaria PfAg*has been accepted by WHO, there is limited data on-field performance other than that in Uganda(18)and its partners in Uganda and Myanmar(19, 20).Therefore, the current study aims to conduct the field performance of the *NxTek*^{TM} *Eliminate Malaria PfAg*test in asymptomatic *P. falciparum*malaria infection in school children in low-transmission environments in Southwest Ethiopia, Jimma Zone, Oromia Region, from September 2021 to January 2022.

1.2. Statement of the Problem

The use of molecular tools is limited to research settings for several reasons, including the ability to distinguish between current and past infections(21). Therefore, the microscope serves as a standard diagnostic tool in the diagnosis of febrile malaria. Malaria RDTs have been used in resource-constrained settings for about two decades(22)**.** The WHO recently reported that global distribution was estimated at 3.1 billion RDTs for malaria diagnosis sold by manufacturers between 2010 and 2020, with more than 81% of sales occurring in sub-Saharan Africa. During the same period, NMPs distributed 2.2 billion RDTs, 88% in sub-Saharan Africa. Among the most commonly used are HRP2 and pLDH antigen-based tests(23). However, reports have revealed a significant rate of false-negative results and an inability to detect low-density parasitemia, severely compromising the malaria elimination program, and the persistence of infections, raising concerns of limiting their diagnostic usefulness(18,20).

The *NxTek*^{TM} *Eliminate Malaria PfAg*Test is not currently used at the Federal Ministry of Health (FMoH) in Ethiopia and is recommended to be used in settings with limited resources for the detection of asymptomatic *P. falciparum*malaria infection(25).

It can therefore be seen that, with the exception of only a few studies evaluating the *NxTek*^{TM} *Eliminate Malaria PfAg*for the detection of *P. falciparum*HRP-II, most malaria RDT performance studies have been primarily aimed at multiple detection methods in febrile malaria infections.

It is, therefore, logical to say that there is a research gap in the performance of *NxTek*^{TM} *Eliminate Malaria PfAg*. Little attention has been paid to the contribution of asymptomatic malaria infection to ongoing transmission, which deserves more research attention. As mentioned above, there is limited research on the performance of an *NxTek*^{TM} *Eliminate Malaria PfAg*detecting *P. falciparum*HRP2 in asymptomatic malaria infections in East African countries including Ethiopia(26).

In addition, WHO (27)has approved this next-generation, highly sensitive HRP2-based *P. falciparum* malaria detection kit, the *NxTek*^{TM} *Eliminate Malaria PfAg*test, for use in malaria diagnosis in populations suspected of having febrile malaria. In addition, the independent evaluation of the WHO prequalification confirmed the performance to detect clinically significant malaria infections (parasitemia>200 parasites/μL) and did not perform the

evaluation of this kit in a population with asymptomatic malaria and presented this issue as a limitation of the committee(27). Based on these facts and other literature-based information as a gap, and taking advantage of the limitations identified in the independent evaluation of the WHO prequalification, the current study was conducted to fill these gaps and evaluate the field performance of the *NxTek*^{TM} *Eliminate Malaria PfAg*in asymptomatic malaria-among schoolchildren in Gomma District, Jimma Zone, southwest Ethiopia.

1.3. Significance of the Study

Asymptomatic malaria infection is a hurdle to malaria control and elimination program. Therefore, detection of asymptomatic *falciparum* malaria infection is a paramount importance for the success of the program. In this regard, *NxTek*^{TM} *Eliminate Malaria P.f Ag* is helpful as a point-of-care diagnosis for detection of *falciparum* parasitemia by the control programs. Hence, the findings of the current study can be utilized by the malaria control programs. This study is also important for healthcare providers who routinely use malaria RDT to detect *p. falciparum* infection. Importantly, an RDT with acceptable clinical performance has the potential to improve the impact of local health departments and public health departments on the malaria control and elimination program by increasing case counts that would otherwise not be achieved with Co-RDTs. The diagnostic performance of *NxTek*^{TM} *Eliminate Malaria P.f Ag*RDT has not been previously performed in Gomma District. Therefore, this study serves as a basis for other researchers interested in performing *NxTek*^{TM} *Eliminate Malaria P.f Ag*services by comparing it to other diagnostic techniques such as microscopy and high-sensitivity qPCR.

CHAPTER TWO: LITERATURE REVIEW

2.1Introduction

Malaria is a mosquito-borne infectious disease caused by the genus Plasmodium. It is transmitted to humans through the bite of infected female Anopheles mosquitoes. Among the top four human malaria parasites, *P. falciparum* (60%) and *P. vivax* (40%) are the most prevalent in Ethiopia(9). It is widespread worldwide, particularly in tropical and subtropical regions, and is more common in sub-Saharan Africa, Asia, and the Americas. Malaria diagnosis plays an important role in supporting the WHO's 2030 Global technical strategy for malaria. Still, the microscope is the gold standard diagnostic tool for malaria infection, but the advent of rapid malaria diagnostic tests (RDTs) in the early 2000s opened up options for malaria diagnosis. Because of its simplicity, speed, inexpensiveness, and usefulness in resource-poor and rural settings without access to laboratory facilities, especially when the microscope is unavailable. But their diagnostic performances are questioned as several factors influence their performance. Therefore, there should be continuous monitoring and evaluation to control their diagnostic performance(28).

The use of molecular tools for malaria diagnosis is limited to research settings for several reasons(29). However, microscopy serves as the gold standard diagnostic tool in malaria-endemic areas, while malaria RDTs have been widely used as point-of-care diagnostics on a limited basis for about two decades(30). The use of RDTs to detect malaria has a major impact on diagnosing the disease. However, its performance can be affected by various factors, especially when the density of the parasite in the blood becomes low. RDTs produce a false negative result(31). This poses a risk to both the community and the countries and may also disrupt the WHO's global malaria elimination program by 2030. Therefore, the use of highly sensitive RDT will reduce the incidence of false negatives.

Recently, the *NxTek*^{TM} *Eliminate Malaria P.f Ag*(with product codes 05FK140, 05FK141, 05FK142, and 05FK143, manufactured by Abbott Diagnostics Korea Inc., CE marked regulatory version) has been approved for the WHO List of prequalified in vitro diagnostic devices(32).It was developed in response to the diagnostic challenges of identifying low-density parasitemia associated with asymptomatic malaria infections in mass screening and active case-detection interventions.

2.2 Diagnostic accuracy of RDTs

There are a large number of published studies, conducted both worldwide and in Ethiopia, describing the performance of malaria RDTs in various areas. For example, in the study conducted in the USA on the diagnostic performance of RDT compared to blood smear microscopy and the use of PCR to verify positive results and discordances, RDT has an excellent performance in detecting *P. falciparum*with 100% sensitivity compared to microscopy with 88% sensitivity. In all malaria diagnostic tests, RDTs were rated superior to blood smear microscopy with a sensitivity of 97%. Comparing the negative predictive value, RDT has 99.6% better performance than blood smear microscopy and blood smear has 98.2%. The authors concluded that mRDT has superior performance for the identification of all *Plasmodium* species than blood smear microscopy(33).In 2011, a similar study was conducted at the China-Myanmar border, comparing the malaria RDT to microscopy for *P. falciparum* identification with a sensitivity of 88.6% and 69.9% for p. vivax with a specificity of 90.4%. However, compared to PCR, malaria RDTs achieve good sensitivity, especially for the detection of *P. falciparum* but has low sensitivity to p. vivax detection. The authors conclude that PCR was highly sensitive than both mRDTs and microscopy. Both RDTs and microscopy are unacceptable results for evidence of mixed infection.Hence, a highly sensitive diagnostic method is needed especially for *P. vivax* infection(34).

Studies have been conducted to perform malaria RDT to detect *P. falciparum*and *P. vivax* can vary depending on the RDT type used. For example in the study conducted in Indonesia(35)a field-based evaluation of Plasmotec Malaria-3 RDT performed well in detecting *P. falciparum* with a sensitivity of 92%) and a specificity of 96.6%. And for the detection of *P. vivax* scored this RDT a performance with sensitivity (66%) and specificity (99.1%) compared to PCR. The author's conclusion suggested that the area for *P.vivax* infection is more common. Even when the RDT met the WHO minimum performance criteria for test specificity (>90%) and <5% invalid rate, the sensitivity of the Plasmotec Malaria-3 RDT was below the test acceptance criteria. Therefore, the sensitivity of this RDT needs to be improved and quality standard microscopy for the detection of *p. vivax* infection is required.

As a global public health goal for malaria control toward elimination programs, the use of HS-RDT plays a significant role, especially in the diagnosis of asymptomatic malaria infection. A study conducted in Tanzania(36)showed a sensitivity of 69% and a specificity of

76.8% comparing HS-RDT with microscopy and a sensitivity of 98.2% and a specificity of 01.6% compared to Co-RDT.

In conclusion, the author concludes that the HS-RDT for *P. falciparum*performed better compared to both microscopy and the currently used malaria RDTs. Therefore, due to its good sensitivity and specificity, the HS-RDT for *P. falciparum*is a better option than the existing commercially available RDT for diagnosing malaria infection(29).

Similarly, the study conducted in the Gambela region on the South Sudan border of Ethiopia (2017) on the prevalence and epidemiological characteristics of asymptomatic malaria based on highly sensitive diagnostics. A cross-sectional study was performed using real-time quantitative PCR as the diagnostic reference method. According to the study, the sensitivity of uRDTAlere Malaria was 33.9% compared to the reference qRT-PCR. But its performance is much better than other diagnostic methods such as CareStart Malaria (14.1%), SD Bioline, and microscopy (5%). In *P. falciparum*specimens only, the sensitivity to uRDTAlere Malaria was 50.0%, higher than SD Bioline (7.3%). The authors conclude that uRDTAlere Malaria has a higher sensitivity than other RDTs and microscopes for detecting asymptomatic malaria. The use of ultra-sensitive diagnostic techniques provides a more accurate result, which has a significant impact on malaria control and elimination programs(30). In addition, in a systematic review including 18 studies, the mean sensitivity of the HS-RDT was 56.1% (95% confidence interval [CI] 46.9 - 65.4%) compared to the co-RDT with a specificity of 44.3% (95% CI 32.6 56.0%) (18). Recently, the performance of HS RDT was evaluated in symptomatic and asymptomatic subjects, and twice as many cases were identified as with co-RDT(20).

The RDTs can perform poorly over other diagnostic methods for a variety of reasons. For example, the study, conducted in North Gondar, Ethiopia, compared the performance of Co-RDTs to nested polymerase chain reaction (nPCR) for diagnosing malaria in public health facilities. Based on the study, CareStartTM Pf/Pan has a sensitivity of 62.9% and a specificity of 92.7%. The authors concluded that the performance of the RDTs was poor compared to nPCR in parasite detection and species identification(37). On the contrary, Alemayehu et al. (2020) reported that the sensitivity and specificity of the HRP2-RDT were 96% and 93%, and those of the pLDH-RDT were 89% and 99%, respectively. The author's conclusion supports its use in the Ministry of Health program and underscored the importance of continuous surveillance to support malaria control and elimination in Ethiopia(38).

Recent developments have shown that improvements are filling the gap. For example, SD BIOLINE Malaria Ag P.f (HRP2/pLDH) is used for the *P. falciparum*screening test to cover the limitations of the current rapid diagnostic test. Like other RDTs for malaria, it is an Immunochromatographic test for the qualitative detection of histidine-rich protein 2 (HRP2) antigen and lactate dehydrogenase (pLDH) on the same strip of human whole blood infected with *P. falciparum*(39). It has been suggested that the performance of RDTs in different malaria-endemic areas and populations needs to be continuously monitored and evaluated. Therefore, for the success of the elimination program, quality-assured malaria RDTs are routinely used in malaria-endemic countries(19).

The newly discovered NxTek Eliminate Malaria Pf, a highly sensitive RDT manufactured by Abbot Diagnostics Korea Inc., has been included in the WHO list of prequalified in vitro diagnostics. For example, the first experimental research by *Slater et al.* (2021), in *P. falciparum* asymptomatic pregnant women in Uganda, compared to conventional RDTs (co-RDTs) when PCR was taken as a reference. The authors showed that the mean sensitivity of HS-RDT was 56.1% compared to 44.3% for Co-RDT(18). Similarly, *Landier et al.* (2018) conducted a field evaluation of its analogs, the ultrasensitive RDTs (uRDT) in eastern Myanmar in asymptomatic malaria-infected individuals. The result showed that HS-RDT showed twice the sensitivity than co-RDTs, 51.4% vs. 25.2%(24).

Another study was conducted to determine the clinical diagnostic performance of two HRP2- and pLDH-based RDTs (SD Bioline HRP2 and CareStartpLDH (pan)) during high and low transmission seasons in Niger. The sensitivity of both tests was >99% during the peak transmission season. However, the authors found a lower specificity of 58.0%. On the contrary, during the low-transmission season, the sensitivity of both tests dropped to 91.0%.

Several studies have also been conducted to determine the performance of malaria in Ethiopia. For example, the study conducted by Getnet and colleagues in north-western Ethiopia(24) showed that the sensitivity and specificity of CareStartTM Pf/Pan were 62.9% and 92.7%, respectively, while the positive and negative predictive values were 76.3% and 87.1%, respectively. In contrast, Alemayehu and colleagues(17) evaluated the performance of Pf(HRP2/LDH) along CareStartMT in the Assosa region, Ethiopia. The authors reported that the sensitivity and specificity of Pf (HRP2/LDH) were 96% and 93%, and that of PfLDH RDT 89% and 99%. Finally, they provided empirical evidence of continued use in the

Ministry of Health's program and underscored the importance of continued studies in support of malaria control and elimination in Ethiopia.

Despite their long clinical/field diagnostic services, *P. falciparum*-based malaria RDTs has encountered several problems in generating false-negative results that impair malaria control and elimination. In recent years, researchers have explored a variety of approaches to answer such questions for future solutions. While false-negative RDTs results have been primarily associated with the test's detection limit, reports have confirmed that genetic variation in *P. falciparum* can also affect the performance of RDTs(40, 41). For example, Schlabe et al. (2021) conducted a case study on a returnee from Ethiopia in Germany. This case study confirmed that the traveler had been diagnosed negative by RDT in Ethiopia and confirmed positive for *P. falciparum*by microscope in Germany. The test result was negative when tested with high quality RDTs: Palutop+4 OPTIMA, NADALR Malaria PF/Pan Ag 4 Species. Finally, the HRP2/3 deletion was confirmed by multiplex PCR(42). the first evidence of P. falciparum strains HRP2/3 gene deletions in the Amazon Basin, Peru(43), expanded reports have been documented inEthiopia(44), Eritrea(40, 45), Kenia(46), Zambia(47), and elsewhere in África(48).

Malaria RDTs are becoming the clinical diagnostic method of choice because of their rapid results and ease of use, even by inexperienced personnel. However, their performance can be affected by several factors that can lead to false positives or false negatives(49). The persistence of the HRP2 antigen in the bloodstream after being free of infection for more than three weeks and the presence of autoantibodies and other infections such as rheumatoid factor (RF) could provide the false-positive result of HS-RDT. A false-negative result can be caused by polymorphism and deletion of the HRP2/3 gene, resulting in the absence of HRP2 expression, the parasite density becomes very high (prozone effect) and the parasite density is very low below the RDT detection capacity threshold. In addition, technical factors also affect the performance of malaria RDTs, such as exposure to high temperatures and humidity that lead to antibody denaturation, improper storage and packaging, poor product design, operator errors, and use of expired kits(49). To determine the sensitivity and specificity of malaria RDTs, continuous monitoring and evaluation is required to control their diagnostic performance in different malaria-endemic areas.

CHAPTER THREE: OBJECTIVES

3.1. General Objective

To evaluate the diagnostic performances of *NxTek™ Eliminate Malaria P.f Ag test*against qPCR for the detection of asymptomatic *P. falciparum* malaria infection among Schoolchildren at Gomma District, Jimma Zone, Ethiopia from September – December 2021.

3.2. Specific objective

- To determine the sensitivity of *NxTek Eliminate Malaria P.f Ag*
- To determine the specificity of *NxTek Eliminate Malaria P.f Ag*
- To evaluate the positive and negative predictive values of *NxTek Eliminate Malaria P.f Ag*
- To assess the prevalence of asymptomatic *falciparum* malaria infection in school children

CHAPTER FOUR: MATERIALS AND METHODS

4.1 Study Area and period

Gomma District: The study was conducted in the Gomma District from September to December 2021: is located 395 km southwest of Addis Ababa and about 45 km west of Jimma town. It's also located at 7° 49' 59.99" N, and 36° 39' 59.99" E, which is among 21 districts in Jimma Zone, Oromia Regional State. It is bounded by SekaChekorsa in the South, southwest by Gera, Northwest by Setema, North by Dhidessa River that separates it from the Illubabor Zone, and in Northeast by LimmuKosa, and East by Mana District. Towns in the district include Beshasha, Choche, Gembe, and LimmuShaye. The Woreda has 42 administrative Kebeles. Agaro is the administrative town of the district. It is found at an altitude that ranges from 1,380 to 1,680 meters above sea level and, according to Danis*oet al.* (2020), its total population is 300,266 of where 152,402are males and 147,864are females; 71,018 or 20.24% of its population are urban residents.Out of 30,514 household heads, 30,247 males and 267 females living in rural Ganda (lower administrative level) of GommaWoreda.Gomma receives an annual average rainfall of about 2000 mm. The climax vegetation in the area is moist Afro-montane forests with characteristic different tree species, as Friis*et al*. (2010) pointed out. Agriculture is the sole source of life for Gomma District rural residents. Of the total 75 schools in the district, five are high schools (9-12) and 70 are primary schools. There are 49 health facilities in the Gomma district t including Agaro General Hospital, 11 health centers, and 37 health posts. In the 42 administrative kebeles of Gomma District, there are 86 active health extension workers with four others on education duties. [Information from Jimma zone Communication, Health and education offices, and Agaro Health office]. Malaria prevention and control in the district are active except bed-net distribution and environmental control are ceased for more than years for unknown reasons. Despite theabove facts**,** Gomma is categorized under a low malaria transmission setting based on its API, according to the district health office.In the district**,** Primaquine is being given as a radical cure for malaria depending on the parasite species and age of the patients. Accordingly, for children aged 5-14 infected with *P. falciparum*, 7.5 mg is given once per oral. And for adults, 15 mg is also given is given one per oral. If children >5-14 are infected with *P. vivax*, 7.5 mg is given orally daily for 14 days while 15 mg is given daily for 14 days if it's an adult. In the district, Primaquine is not given for under-five children.

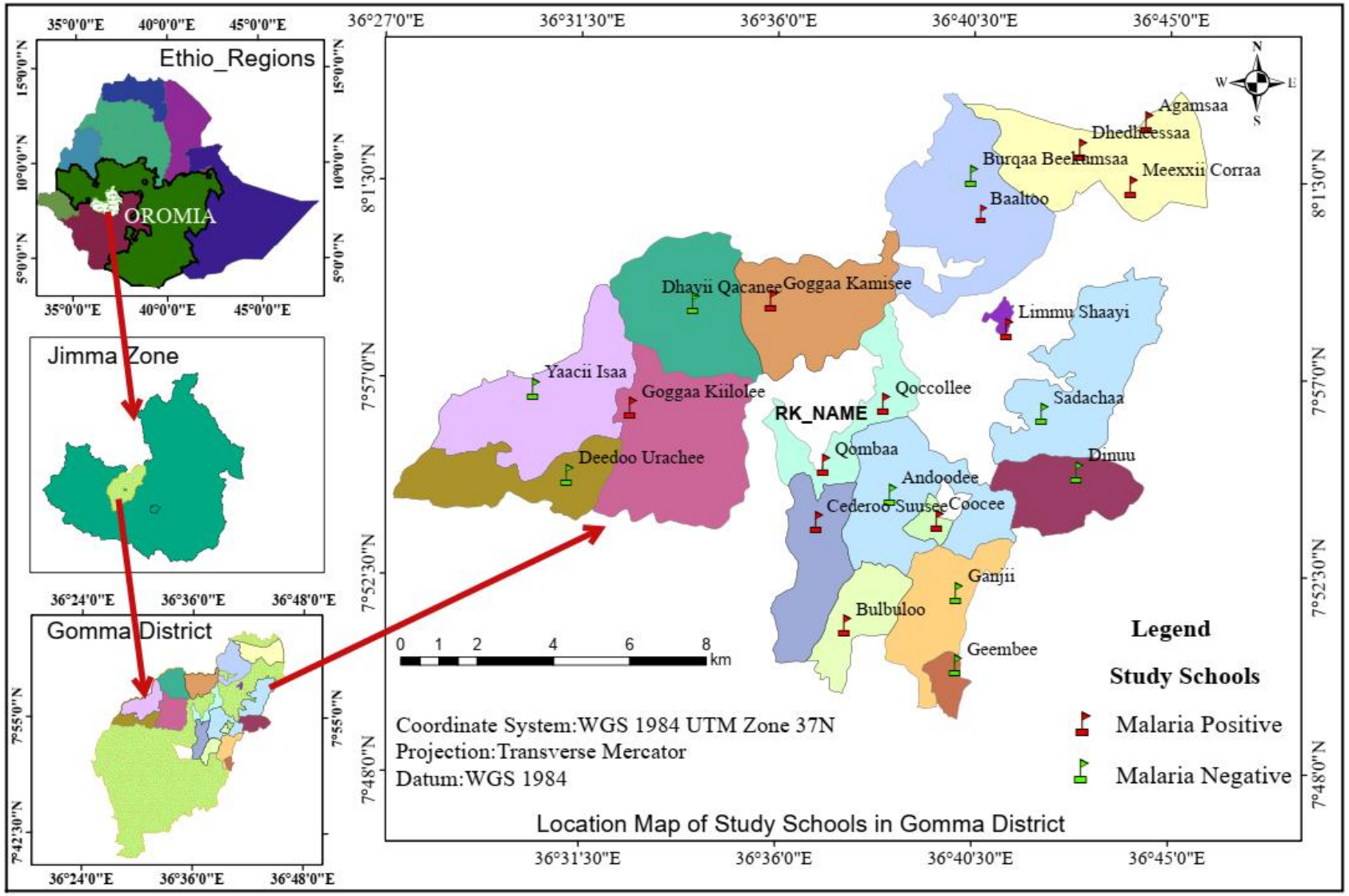

Figure 1: Map of selected study primary school in Gomma district, Jimma Zone

4.2 Study Design and Period

- The school-based cross-sectional study design was conducted from September to December 2021

4.3 Population

4.3.1 Source Population

- All School children who were aged 5-15 years old at selected primary schools

4.3.2 Study Population

- All family/guardians consenting school children 5-15 years old at selected primary schools

4.4 Sample Size determination and sampling technique

4.4.1 Sample size determination

The sample size for study participants with asymptomatic malaria was calculated based on the following assumptions:

A. The first assumption: - A single population proportions formula is used to determine the sample size. P is the prevalence of asymptomatic malaria 6.8% in school children(50),95% confidence interval (CI), and d is the margin of error (precision), assumed to be 2% (to maximize the sample size for such rare cases).
B. The second assumption is that the malaria exposure risks were different in the Gomma district kebeles and therefore the design effect of 1.5 is assumed (feasibility issues were taken into account).Accordingly, the following formula was used:

$$n = \frac{\left(z\frac{\alpha}{2}\right)^2 p(1-p)}{d^2}$$ Where n = the sample size, z = 1.96

Equation 1: Formula for calculating samples size

Accordingly, the calculated sample size (n) was 608.66. Adding a non-response rate of 10% gave a total sample size of 668. Since it was assumed that there was a difference in malaria exposure among the Kebele, we take the design effect of 1.5, i.e. n = 668 * 1.5 = 1002 students registered in the study.

4.4.2 Sampling technique

A multi-stage sampling technique was used. That is, Gomma district was randomly selected as the primary sampling unit from the list of 21 districts in the Jimma zone. Of the 70 elementary schools (1st to 8th grade) in the district, 21 were also systematically selected. Schools were also selected as secondary sampling units. Finally, the sample size was proportionally allocated to the individual elementary schools and then to the individual grades and sections. Therefore, each child was selected as the final sampling unit using the lottery method from lists of student records and stratified by age (5 to 15) and grade level, following protocols developed by Brooker and colleagues(51, 52)and methods used by*Worku et al.*(50).

In summary:

$$\text{Proportional allocation} = \frac{Number\ of\ students\ in\ class\ \text{X}}{\textbf{total no of students in the selected school}} * \text{Total sample size}$$

Equation 2: Formula for calculating proportional allocation

4.4.3 Sampling frames

- The sampling frames lists of districts from Jimma zone, lists of schools in Gomma district education office and the registry book(roster) at each school for each classsampling technique used wasa systematic random sampling technique from the sampling frames.

4.5 Inclusion and Exclusion Criteria

4.5.1 Inclusion Criteria

- All consenting school children who are 5-15 years old and who were at school during data collection
- A school child who didn't take anti-malaria treatment the previous month
- A student whose body temperature was <37^{o}c
- School children who have been residents of the area for more than six months

4.5.2 Exclusion Criteria

- School children who were taking anti-malaria drugs within a month
- School children who were febrile
- Children whose parents or guardians refused to provide accent
- Students who were older than 15 years of age

4.6 Study Variables

4.6.1 Dependent Variable

- Sensitivity
- Specificity
- Positive predictive Value
- Negative predictive value

4.6.2 Independent Variables

- Age
- Sex

4.7 Operational Definitions

- **School children**: children who are 5-15 years of age and at a primary school on education
- **Asymptomatic malaria**: a *Plasmodium* parasite infection in which a school child shows no sign and symptoms
- **Microscopic asymptomatic malaria**:detection of *Plasmodium* parasites from a finger pricked Giemsa- stained blood smear bymicroscopy from healthy school children
- **Submicroscopicasymptomatic malaria**: detection of DNA of *Plasmodium* parasites from DBS by qPCR that light microscope unable to detects due to their low density in the blood circulation
- **Febrile Individuals:** are individuals the temperature is above 37^{o}C

4.8 Data Collection Materials

- RDT kits
- Microscope
- Frosted microscope slides
- Cotton
- Glove
- 70% alcohol
- Watmann filter paper 2
- Pen
- Pencil
- Pencil-sharpener
- A4 paper
- Permanent marker

4.9 Laboratory Sample Collection and Analysis

4.9.1 Blood sample collection and microscopic examination

After demographic data collection, finger prick blood samples were taken from each study participant at each study school. Drops of finger-pricked blood were collected for on-site testing by *NxTek*^{TM} *Eliminate Malaria PfAg*and Co-RDT. Dried blood spots (DBS) were collected for qPCR assay. Thick and thin blood films were also prepared on the same frosted-end microscopic slides and stained with 10% buffer-diluted Giemsa stain for 10 minutes and examined through a 100X oil immersion objective. Species identification and parasite density were determined by experienced laboratory technologists. A negative slide was declared after 100 fields had been exhaustively examined(51). For the Parasite counts, blood stage parasite densities were determined using the standard WHO formula(53). That is, the parasite count was performed against 500 white blood cell (WBC) counts, assuming a mean WBC count of 8,000/L. Identification of the Plasmodium parasite species was made by examining a thin blood smear using the same lens. Quality control was performed at every step of the process. A blood smear was read independently by two qualified microscopists, each blinded to the other reader's results and results. Conflicting results were resolved by a third experienced microscopists who would be blind to the first two microscopists, as previously described in the reference above(53).

4.9.1.1 Quality control of laboratoryworks

Before the actual laboratory tests, the prepared Giemsa stain was compared with commercially available Giemsa solutions and the qualities were compared. Finally, based on the standard WHO protocol(37), the following key parameters were met for our Giemsa stain prepared from Giemsa powder (AppliChem Chemical Synthesis Service, Cat. No. 51811-82-62, product date 2018, Germany) was manufactured by Ethiopian Pharmaceutical Supply Agency (EPSA) branch Jimma:

Quality checked on parasite free blood films:

- **Thick blood film** – clear white blood cell cytoplasm stained blue and nuclear granules stained purple
- **Alcohol-fixed thin blood film**: clear visualization of platelets, white blood cell cytoplasm stained blue, and nuclear granules stained purple
- **Red Blood Cells (RBCs):** Size and shape

Quality checked onKnown parasite positive blood films

- **Parasite detection on thick blood film:** clear parasite cytoplasm(deep blue or faint), red chromatin dots(one or two: amoeboid or compact)
- **Parasite detection on thin blood film**: infected RBCs sizes, whether it contained parasite(s) or not. Then, cytoplasmof the parasites, chromatin dots and number of parasites within RBCs

4.9.1.2 Quality control of Microscopic examinations

Three expert microscopists, blinded to the positivity of the slides and the purpose of the study, examined the slides and approval was obtained by the third examiner in the event of conflicting results. Parasite counts normally performed for asexual stages of Plasmodium species are performed based on the standard protocol given above. Accordingly, the presence of gametocytes and asexual parasite stages has been reported. Upon completion of the parasite density count, positive slides were mounted using D.P.X-Mountant and saved for future studies.

4.9.2 The ImmunochromatographicMalaria RDTs

4.9.2.1 The *NxTek™ Eliminate Malaria PfAg*

A two-band *NxTek™ Eliminate Malaria PfAg*test with product codes 05FK140, 05FK141, 05FK142 and 05FK143 manufactured by Abbott Diagnostics Korea Inc. CE marked regulatory version (WHO reference number: PQDx 0349-012-00) is describe a qualitative and differential test for the detection of *P. falciparum* HRP2 in human whole blood (32, 54).The test is intended for professional and point-of-care testing to aid in the diagnosis of malaria infection in a general population, including pregnant women. The RDT contains a membrane strip pre-coated with mouse monoclonal antibodies specific for the *P. falciparum* HRP2 in the region of the test band. This test device has a letter of C and *P.f*. The test line represents the control line while the *P.f* test line is the line test for *P. falciparum*HRP-II. Both test lines in the result window are not visible before applying samples(54). The general procedures are shown according to the manufacturer's instructions (Annex V).To ensure the quality of data before the actual field work the *NxTek™ Eliminate Malaria PfAg*was checked by known positive *P. falciparum* blood sample at least for five kits.

4.9.2.2 The Conventional Rapid Diagnostic Technique (Co-RDT)

A triple band (two test lines, one detects only HRP2 of *P.falciparum* and the other pLDH of *P.vivax*, and control band) Conventional Malaria RDT, SD Bioline Malaria Ag P.f/P.v Product date: 07/13/2020 and Expiry date: 07/12/2022, Republic of Korea) was also used against *NxTek*^{TM} *Eliminate Malaria PfAg*, the microscope and qPCR. To check the quality of Co-RDT before the actual field work the Co-RDT was checked by known positive *P. falciparum* blood sample at least for five kits.

4.9.3 The molecular assay

4.9.3.1 Extraction of Parasite DNA from DBS

Parasite DNA was extracted from DBS using the Chelex-saponin method (Annex V) as previously described(55). In this procedure, the TaqMan probe was used to detect both *Plasmodium* parasite species-specific assays. A qPCR machine, QuantStudio3 Real-Time Multiplex PCR Instrument (Thermo Fisher Scientific) was operated to amplify the 18S rRNA genes for detection of the indicated *Plasmodium* species using a pair of forward and reverse primer sequences and probes(Table 1).

Table 1: TaqMan probe detection of plasmodium parasite Species-Specific detection

ref.	Species	Primer or Probe*	Sequence (5'-3')	Length(bp)
(56)	*P. falciparum*	F-F(forward)	TATTGCTTTTGAGAGGTTTTGTTACTTTG	
		F-R (Reverse)	ACCTCTGACATCTGAATACGAATGC	221
		Pf-fam (MGB)	ACGGGTAGTCATGATTGAGTT	
(57)	P. vivax	Pv-1 (Forward)	CGCTTCTAGCTTAATCCACATAACTG	142
		Pv-2 (Reverse)	AATTTACTCAAAGTAACAAGGACTTCCAAG	
		Pv-probe (VIC-MGB)	CGCATTTTGCTATTATGT	

Quantitative PCR amplification was performed in the total reaction volume of 12µl containing 1.4µl molecular grade water, 6µl PerfeCTa master mix, 1.6µl each of forward and reverse primer, 0.5µl each of probe for P falciparum (Pf-Fam) and contained P. vivax (Pv-vic) and 2µl extracted DNA under the following PCR cycling conditions for 45 cycles (table 2)

Table 2: Total running time of qPCR assay

Check the cycling conditions Temperature (ºC)	Time	Number of cycles
50	2 minutes	45
95	2 minutes	
95	3 seconds	
60	30 seconds	

NB: Protocol adapted from Molecular Biology Laboratory, International Centers of Excellence for Malaria Research (ICEMR), Jimma University Tropical Infectious Diseases Research Center (TIDC), Sokoru, and Jimma.

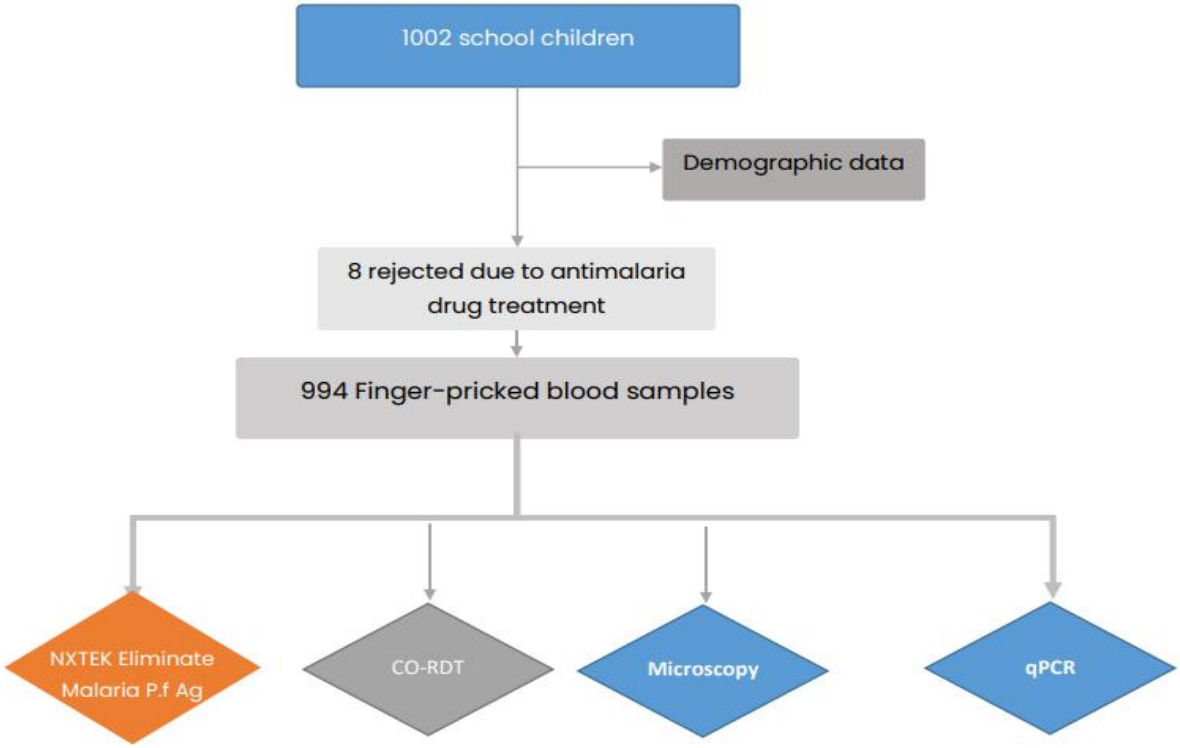

Figure 2: Study flowchart for malaria RDTs, the microscopy, and qPCR assay

4.10 Data Quality Management

Pre-analytical, analytical and post-analytical precautions were taken to ensure the quality of the data. For the demographic data collected, the English version of the consent form was translated into Afaan Oromo (Amharic) and translated back to the English version for its accuracy and consistency. Manufacturer's instructions and standard operating procedures (SOP) were followed during sample collection and all other laboratory procedures. In addition, data collectors were trained to focus on taking blood samples (for microscope, RDTs, and DBS), operating the RDTs, the sample process, pipetting for RDTs equipment, and examining stained blood smears and RDTs tests(58, 59).Finally, a log book was prepared and all results were recorded along with each student's identification number (code).

4.11 Statistical Analysis

Data were entered using Epi-data version 3.1 and analyzed using Statistical Package for Social Sciences (SPSS) version 26. The sensitivity, specificity, PPV and NPV were calculated against the reference method. Sensitivity was calculated as the ratio of positive test results to true positives; Specificity was calculated as the ratio of negative test results to true negatives. The positive predictive value was calculated as the proportion of true positives among all positive-reacting specimens, and the negative predictive value was calculated as the proportion of true negatives among all negative-reacting specimens. The accuracy of the test method was also calculated. Proportions were compared using the chi-square test. The 95% confidence interval was taken for each parameter. The agreement between these malaria diagnostic methods was assessed using the kappa value.

4.12 Ethical Consideration

Ethical clearance was obtained from the Research and Ethical Review Committee/Institutional Review Board (IRB) at Jimma University, Institute of Health. A letter of support from the Institute for Health Research and Innovation at the University of Jimma was submitted to the Jimma Zone Health and Education Offices. Letters of approval were then written to Gomma district health and education departments. Final letters were sent to the selected primary schools and local health authorities. Final permission was then obtained from each school child's families/guardians. The data collectors were read the aim of the study to the families/guardians of the study participants from submission to those who can read and understand the information sheet, which was prepared in the participants' language. In addition, written informed consent was obtained from the parents/guardians of the study participants

prior to data collection. Participation in the study was voluntary and the full autonomy of the study participants was respected. To ensure data confidentiality, study participants were identified using codes.Positive results were communicated to the family/guardians of students and then given the standard of care at nearby health centers or health extension works accompanied by lists of students, age, sex, and grades.

4.13 Dissemination of the Result

The results of this study will be presented to the Jimma University Postgraduate School, Institute of Health, Faculty of Health Sciences, School of Medical Laboratory Science and Gomma District Health Department. The results of the work will be presented as a public defense at Jimma University. More importantly, the results of the current project will also be presented at scientific conferences and published in a peer-reviewed international journal.

CHAPTER FIVE: RESULTS

5.1. Characteristics of study subjects

A total of 1002 school children (5-15 years old) took part in the study. Eight students were rejected due to antimalarial drug treatment, bringing a total of 994 students who participated in the study(Figure2). Accordingly, 51.9% (516) were males while 48.1% (478) were females. The mean age of the study participants was 11 years. The current finding revealed that asymptomatic malaria was 6.4% (33/516) higher in males than in females, 3.5% (17/478). The result shows that grades 1-4 have a high burden of asymptomatic malaria infections (Table 3). The majority of participants (40%) had parasite densities in the range of 192-1984 parasites/L (Table 4). On the other hand, the age groups of 10-15 years had the highest parasite density and were higher in males than in females.

Table 3: *P. Falciparum* positivity rate using RDTs, Microscope, and qPCR among school children at Gomma district, southwest Ethiopia, September - December2021

Demographic characteristics	Study participants (n)	Diagnostic tools		
		NxTek™/ Co-RDT (n/%)	Microscopy (n/%)	qPCR (n/%)
Gender				
Male	516	19 (3.7)	12 (2.3)	34 (6.6)
Female	478	3	3	16 (3.3)
Age group				
5-9	263	7	3	16 (6.1)
10-15	731	15 (2.0)	12 (1.6)	34 (4.6)
Grade				
1-4	285	13 (4.6)	8	27 (9.5)
5-8	709	9	7	23 (3.2)

5.2. Prevalence of asymptomatic malaria

The overall prevalence of asymptomatic malaria in school children was 5%. The prevalence of *P.falciparum* was 4.5% by qPCR, 2.2% by *NxTek*^{TM} *Eliminate Malaria PfAg*/Co-RDT, and 1.5% detected by microscope. Of the total prevalence, *P. vivax* was detected in 10%(5/50) while the mixed infection was detected in 6%(3/50) of the cases(Fugue3).

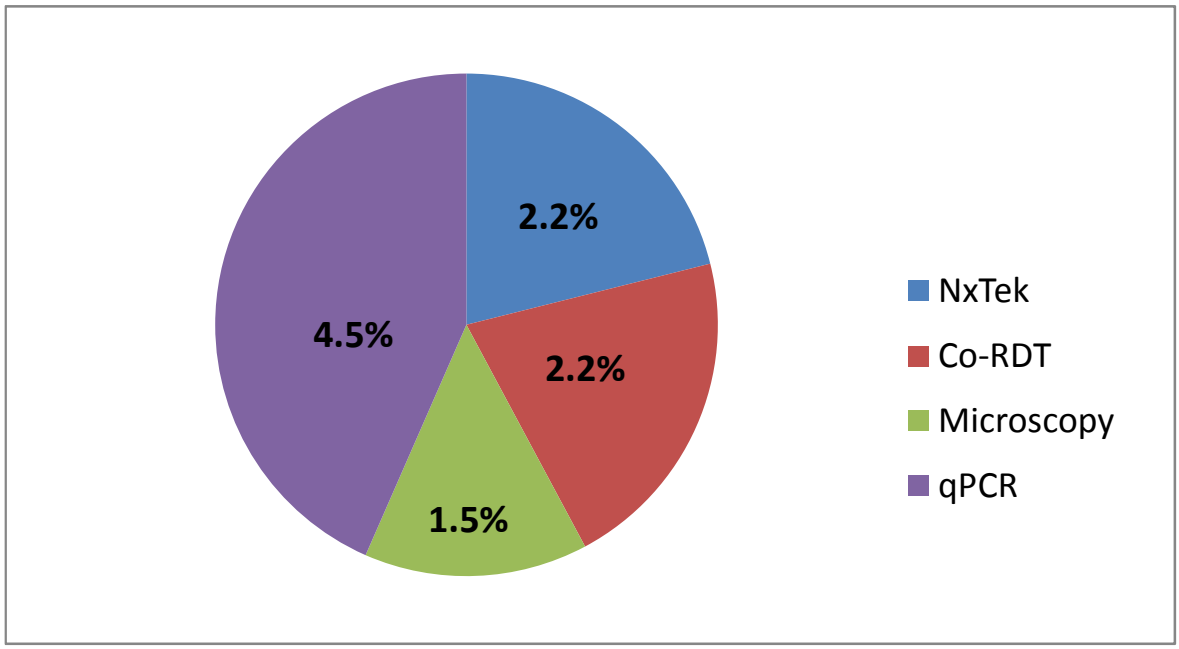

Figure 3: Prevalence of asymptomatic malaria in school children using different diagnostic tools at Gomma district, Jimma zone from September –December 2021.

Table 4: Distribution of parasite density by sex and age at Gomma district, southwest Ethiopia, September -December 2021

Parasite density/μL	Study participants			
	Gender		Age group in years	
	Male (%)	Female (%)	5-9 (%)	10-15 (%)
64 - 96	2 (13.3)	2 (13.3)	_	4 (26.7)
192- 1984	5 (33.3)	1 (6.7)	1(6.7)	5 (33.3)
2224 - 23440	5 (33.3)	_	2 (13.3)	3 (20)

5.3. Diagnostic performance of *NxTek™ Eliminate Malaria PfAg*

Compared to the gold standard qPCR, the sensitivity of *NxTek™ Eliminate Malaria Pf Ag*was 44% (95% CI 29.99% to 58.75%), while the specificity was 100%. (95% CI 99.61% to 100%). PPV and NPV were 100% (95% CI 99.61% to 100%) and 97.43% (95% CI 96.74% to 97.98%), respectively. Thediagnostic accuracywas found to be 97.48% (95% CI 96.30% to 98.36%)The kappa value was reported as 0.62 indicating substantial agreement between the methods**.**

Table 5: Diagnostic performance of *NxTek™ Eliminate Malaria Pf Ag* compared to qPCR for the detection of asymptomatic *P. falciparum* malaria at Gomma district, Ethiopia, Sept - Dec 2021.

Test		qPCR		Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	Kappa (95% CI)
		Pos	Neg	44% (29.9, 58.7)	100% (99.6,100)	100% (99.6,100)	97.4% (96.7,97.9)	0.62
NxTek™	Pos	22	0					
	Neg	28	944					

Comparedto the conventional malaria diagnostic tool, the microscope, the sensitivity of *NxTek™ Eliminate Malaria PfAg*was 68.18% (95% CI 45.13% to 86.14%), while the specificity was 100%. (95% CI 99.62% to 100%). PPV and NPV were 100% (95% CI 99.61% to 100%) and 99.52% (95% CI 99.11% to 99.74%), respectively. The accuracy of the test was 99.52% (95% CI 98.87% to 99.85%). The diagnostic agreement between the *NxTek™ Eliminate Malaria PfAg*and the microscope was 0.81 showing the perfect agreement.The *NxTek™ Eliminate Malaria PfAg*was also compared with CO-RDT and was found to be similar findings, hence results are presented here as one RDT in this context across this document.

Table 6: Diagnostic performance of NxTekTM Eliminate Malaria Pf Ag compared to Microscopy for the detection of asymptomatic P. falciparum malaria at Gomma district, Ethiopia, Sept - Jan 2021

Test		Microscopy		Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	Kappa (95% CI)
		Pos	Neg	68.2% (45.1,86.1)	100% (99.6, 100)	100% (99.6,100)	99.52% (99.1,99.7)	0.81
NxTek™	Pos	15	7					
	Neg	0	972					

CHAPTER SIX: DISCUSSION

The foremost difficulties with the existing malaria RDTs are the inability to detect low-density circulation malaria parasites and results in false-negative outcomes. Continuous field-based performance evaluation of malaria RDTs has paramountimportance for monitoring and boosting the current global malaria elimination programs. In this regard,recently, theWHO(27) has approved the next-generationhighly-sensitive HRP2 based *falciparum* malaria detecting kit, the *NxTek™ Eliminate Malaria P.f Ag test* for use in the malaria diagnosis in febrile malaria suspected population. In addition to this, the WHO prequalification independent evaluation verified performance to detect clinically significantmalaria infection (parasitemia>200 p/μL) and didn't perform the evaluation of this kit in the asymptomatic malariapopulation and put this issue as the limitation of thecommittee(27). Based on the above facts as information gap and taking over the advantage of the limitations stated by the WHO prequalification independent evaluation, the current studywas carried out to seal the gaps and aimed at evaluating the field performance of this kit among school children in Gomma district, southwest Ethiopia.To our knowledge, the study reported here is the first of its kind in Ethiopiawherenew malaria RDT was evaluated in healthyschool children and provides a keycontribution to malaria control followinginfection.

The current finding presented here shows that the detection rate of *NxTek*^{TM} *Eliminate Malaria PfAg*for *P. falciparum* in apparently healthy school children (asymptomatic) was lower (22/994) than qPCR (45/994). Therefore, qPCR detected 51.1% (23/45) more *P. falciparum* than *NxTek*^{TM} *Eliminate Malaria PfAg*, showing that qPCR detected parasites at a very lower density than *NxTek*^{TM} *Eliminate Malaria PfAg*. This shows that the *NxTek*^{TM} *Eliminate Malaria PfAg*false-negative rate was 51.1% (23/45). Therefore, the difference could be due to the detection threshold of the diagnostic tools.
Compared to qPCR, the sensitivity of the *NxTek*^{TM} *Eliminate Malaria PfAg*is 44%, which is similar to the study conducted in a low transmission environment in Myanmar (44%) but lower compared to the study conducted in a high malaria transmission setting in Uganda (84%). This is because the similarity of the results with those in Myanmar could be due to a similar malaria transmission setting as in the Gomma District, unlike in Uganda since the study was conducted in a high malaria transmission setting(63). The other reasons could be geographic characteristics of study settings and seasonality. And the specificity was 100% which is higher than the study conducted in Assosa with aspecificity of 93% (38).

Compared to microscopy, the sensitivity and specificity of *NxTek*^{TM} *Eliminate Malaria PfAg*are 68.2% and 100% which is very high compared to the study conducted in Nigeria which scores 42.5% and 87.1% respectively (61). The difference in the result could be due to geographic characteristics of study settings and seasonality. showed a positivity rate of *P. falciparum* (1.5%; 15/994) and a positive predictive value 100% which is very high compared to the study conducted inGambela region, and Nigeria (60, 61). These results confirmed the ability of *NxTek*^{TM} *Eliminate Malaria PfAg*to correctly identify *P. falciparum* malaria-infected individuals as truly positive and *P. falciparum* malaria-freeindividuals as a true negative. Furthermore, the negative predictive value was 68.2%. These findings are conducted previously in Ethiopia, Assosa (31), Mozambique(45), and Uganda (46).

Compared to the Co-RTD, the *NxTek*^{TM} *Eliminate Malaria PfAg*showed similar performance for the diagnosis of Asymptomatic *P. falciparum*malaria infection. This finding is different from the study conducted in Eastern Myanmar among asymptomatic patients with a sensitivity of uRDT (51.4%) and Co-RDT (25.2%) with similar specificity 99.5% for uRDT and 99.9% Co-RDT.However, we observed that some of the tests showed very faint positive band lines for Co-RDT while all were visible and a deep navy blue line for positive *P.f* on *NxTek*^{TM} *Eliminate Malaria PfAg*. This indicates that as the circulating HRP2 concentration decreases, the probability of missing Co-RDT is higher, while *NxTek*^{TM} *Eliminate Malaria PfAg* could still be positive. Therefore, it is suggested that in this regard, the *NxTek*^{TM} *Eliminate Malaria PfAg*is a more preferable malaria diagnostic tool than the co-RDT for *P. falciparum*. Therefore, lower circulating HRP2 means a lower density of circulating parasites; hence *NxTek*^{TM} *Eliminate Malaria PfAg*has the potential to capture fewer circulating parasites than Co-RDT. Accordingly, the *NxTek*^{TM} *Eliminate Malaria PfAg*can alternatively be used as co-RDTs in support of the malaria elimination program in endemic countries.

On the other hand, *NxTek*^{TM} *Eliminate Malaria PfAg*showed a superior detection rate of 2.2% (22/994) of asymptomatic *P. falciparum* malaria than microscope 1.5% (15/994) in children. That means it detected a 0.7% higher detection rate of asymptomatic malaria than the microscope. Therefore, this result suggests that *NxTek*^{TM} *Eliminate Malaria PfAg*could be used for public malaria screening and for malaria diagnosis in lower-level health facilities where a microscope is not affordable.

The overall prevalence of asymptomatic malaria in Gomma district was 5%, which is lower than the study conducted on asymptomatic school children (6.8%) in Gondar, Ethiopia (50)and in North Shoa, Ethiopia, where the prevalence of asymptomatic malaria in pregnant women was 5.7%(62). The difference may be due to differences in sample size, diagnostic tools used, and subjects of study (in North Shoa).

The prevalence of*P. falciparum* was 4.53% (45/994), which is lower compared to the study conducted in Uganda using the *NxTek*^{TM} *Eliminate Malaria PfAg*in asymptomatic school children which were 21% (20). The difference in detection rate could be due to differences in the occurrence of parasites in Uganda, the timing of data collection, and seasonal variations in malaria incidence in the region. In our finding, the prevalence of *P. falciparum* is lower compared to a study conducted in the Assosa zone(38) using PfHRP2 detecting RDT(24.1%) in the febrile population. In the current study, the infection rate of *P. falciparum* was higher in males(6.6%) than in females (3.6%) that females which is similar tothat of the study conducted in Assosa(38) and a study conducted in Gondar(50).

In the current study, the qPCR-based prevalence of *P. vivax* and mixed infection was 10% (5/50) and 6% (3/50), respectively, but no other diagnostic tools used were identified. Our result showed that the majority of participants (40%) had a microscopic parasite density ranging from 192 to 1984 parasites/μL. Age groups of 10 to 15 years had the highest parasite densities because children at this age have slightly higher parasite densities compared to adults and the immune response in children is much more aggressive than in adults(36).

6.1. Limitation of the study

1. We did not evaluate the performance of the *NxTek*^{TM} *Eliminate Malaria PfAg*in symptomatic individuals.
2. We did not investigate the possible cause of false-positive and false negatives results
3. We did not perform parasite density count by qPCR

CHAPTER SEVEN: CONCLUSION AND RECOMMENDATIONS

7.1. Conclusion

The sensitivity of *NxTek*^{TM} *Eliminate Malaria P.f Ag* compared to qPCR was low. It could possibly due to high prevalence of HRP2/3 gene deletions, as evidenced from literatures in the country. Hence, we fell that the difference comes from such deletions and might have jeopardized the exact test comparisons among the diagnostic tools used. On the other hand, both *NxTek*^{TM} Eliminate Malaria P.f Ag and Co-RDT showed similar results; again it could be an indicative of the gene deletions where both HRP2 positive RDTs detected the undeleted HRP2 in positive samples. Therefore, *NxTek*^{TM} *Eliminate Malaria PfAg* can be used as an alternative diagnostic tool for *P. falciparum* malaria infection. On the other hand, the 5% prevalence of asymptomatic *P. falciparum* malaria infection among schoolchildren in Gomma district of Jimma zone indicates an alarmingly active malaria transmission in the area.

7.2. Recommendations

The authors of the current study recommend further studies on the symptomatic *falciparum* malaria populationand pregnant women. Additionally further study on the PfHRP2/3 gene deletion for possible false-negative results. For Policy/Malaria Control Program: According to Jimma Zone Malaria Control Office data, Gomma District is an area of low malaria transmission. The present study provides an alarming finding of active malaria transmission among schoolchildren in the area. The authors of this work recommend the malaria control program to strengthen intervention strategies. One of these strategies is point-of-care diagnostics. More importantly, the cost of both *NxTek*^{TM} *Eliminate Malaria P.f Ag*is similar to that of the Co-RDT. Therefore, the *NxTek*^{TM} *Eliminate Malaria P.f Ag*can be used as routine *P. falciparum* malaria diagnostics in resource-constrained areas due to its thermal stability (1-31°C), cost-effectiveness, and ease of use in low-level trainees. In addition, the Ethiopian Ministry of Health needs to consider including the *NxTek*^{TM} *Eliminate Malaria P.f Ag*in the malaria diagnostic algorithm to enhance the malaria elimination program.

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ANNEXES

ANNEX I: Participant information sheet

Part IA: Information sheet: English version

Title of the Research: To evaluate the diagnostic performances of *NxTek™ Eliminate Malaria P.f Ag test*against qPCR for the detection of asymptomatic *P. falciparum* malaria infection among Schoolchildren at Gomma District, Jimma Zone, Ethiopia from September – December 2021.

Name of Principal Investigator: Ashenafi Demeke (BSc, MSc candidates)

Advisors: Prof. Delenasaw Yewhalaw (PhD)

Mr. Abdissa Biruksew (MSc, PhD fellow)

Name of the Organization: Jimma University, Institute of Health, Faculty of Health Sciences, School of Medical Laboratory Sciences.

Introduction: This information sheet is prepared with the aim of explaining the research that you are asked to join as a research participant. This information sheet describes the research.

Aim of the study: To evaluate the diagnostic performances of *NxTek™ Eliminate Malaria P.f test*against qPCR for the detection of asymptomatic *P. falciparum* malaria infection among Schoolchildren at Gomma District, Jimma Zone, Ethiopia from September – December 2021.

Procedure: If the family is agreed to take part his/her child in the study, the Laboratory professional will give verbal and/or written information about the study, and the family/guardian will sign the consent form. Students are kindly requested to give the correct information about themselves and the necessary measurements were performed by the assigned Laboratory professional.

Risk and discomfort: Participating in this research will not cause more discomfort than is required you could go through for routine examination. There may be slight pain during blood sample collection. If there is any discomfort like swelling at the site of blood drowns, we shall offer you the necessary medical treatment freely. One drop of blood taken from each volunteer throughout the study period will not affect your health.

Benefits: If you are participating in this study, youwill be treated and your participation is likely to help us an important input to evaluate the diagnostic performance of *NxTek™ Eliminate Malaria P.f Ag*.

Incentives and payment for participating in the study: You will not be provided with any direct incentives for your participation in this study. But the cost of your medical examination will be covered.

Confidentiality: All information about the study participants will be kept confidential. The name of the participant will be coded. The information sheet that links the coded number to the participants name will be locked inside a computer and it will not be revealed to anyone except your nurses and the principal investigator.

Right to refuse or withdraw: You have full right to withdraw from participating in the study at any time before and after consent without explaining the reason and not responding to some or all the questions. Your decision will not affect your right to get the health service you are supposed to get otherwise.

Contact Address

If you have any questions or concerns, you can contact Ashenafi Demeke at any time using the following address:

Ashenafi Demeke, Medical Parasitology MSc student at Jimma University, Institute of Health, Faculty of Health Sciences, School of Medical Laboratory Sciences.

Prof. Delnesaw Yewhalaw (0917804352)

Mr. Abdissa Biruksew (0911964174)

Tel: 09-17-00-41-80

09-34-45-34-24

Email: ashudemeke2014@gmail.com

Jimma, Ethiopia

Thank you very much!!

Part IB: Information sheet: AfaanOromoversion

Mata dureeqorannoo: JiraattotanaannooZooniiJimmaaaanaaGommaaworredaairrattidandeettiimeeshaadhukkubabusaayaaluuf gargaarumadaaluu.

Maqaaqorataaqorannichaa: AshannafiiDammaqaa (BSc, MSc candidates)

Gorsaa: Prof. DilnessaawYewaahalaw (PhD)

Mr. AbdiisaaBiruuksew(MSc, PhD fellow)

Maqaadhaabbatichaa: Jimmaayuuniversiitii, Inistituutiifayyaa, Faakaaltiisaayinsiifayyaa.Kutaabarnootaameedikaallaaboraatootiisaayinsii

Seensa: Bocniodeeffannookanaakanqophaaekaayyooqorannookanaanamaqorannoo kana keessattihirmatuufibsagahaakennuudhaafi.

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Mirgaqorannookeessaabahuu: Yeroobarbaaddeettiqorannichakeessaabahuufmirgaguutuuqabda. Murteenkeessantajaajilafayyaaargachuukeessaanirrattidhiibbaahinqabu.

Teessoo

GaaffiikamiyyuuyooqabaattanyeroobarbaaddanAshanaafiiDammaqaawaliinhaasa'uunidandeessu.

AshannaafiiDammaqaa, MeedikaalParasitooloogiiBarataa MSc Jiimmaayuuniversiitii, Instituutiifayyaa, Faakaaltiifayyaasaayinsii, KutaabarnootaaMeedikaal Laaboraatooriisaayinsiiciences.

Lakk.Bil.:

09-17-00-41-80

09-34-45-34-24

Email: ashudemeke2014@gmail.com

Jimma, Ethiopia

Part IC: Information sheet: Amharic version

የጥናቱርዕስ: በጅማዞንበጉማወረዳአካባቢበሚኖሩሰዎችላይየወባበሽታመመርመሪያመሳሪያአቅምንየመመርመርአቅምሰለማጥናት

የተመራማሪስም፡- **አሸናፊደመቀ**፤የድርጅቱስም፡- ጅማዩኒቨርስቲ / የህክምናላብራቶሪሳይንስት /ቤት

የአማካሪዎችስም፡- **ፕሮፌሰርድልነሳውየወሀላው**

አቶአብዲሳብሩክሰው (የዶክትሬትተማሪ)

መግቢያ፡- ይህየተዘጋጀውበአጭሩሰለሚካሂደውጥናትለጥናቱተሳታፊዎችበጥናቱጉዳዩሃሳብለማካፈልነዉ፡፡ ሰለሆነምየዚህጥናትዋናአላማበጅማዞንበጉማወረዳአካባቢበሚኖሩሰዎችላይየወባበሽታመመርመሪያመሳሪያንየመመርመርአቅምሰለማጥናትእናየበሽታዉንማህበራዊእናኢኮኖሚያዊጉዳትለመቀነስየዚህጥናትውጤትየበኩሉንሚናይጫዎታል፡፡

የጥናቱሂደትዝርዝር

በጥናቱለመሳተፍከተሰማሙየሚከተሉትንመረጃዎችእናናሙናእንወሰዳለን::
ከእርሶየደምናሙናተወሰዶምርመራይደረጋልየተሰበሰበውናሙናጅማዩኒቨርስቲላብራቶሪከጥናቱጋርየተያያዙየወባበሽታምርመራይደረጋል

ስጋትእናጉዳት

በአጠቃላይከላይየተጠቀሰዉንናሙናበሚወሰድበትጊዜበናሙናመወሰጃመርፌአነስተኛየሆነጊዜያዊህመምውጪሌላምንምችግርእንደማያጋጥምየተረጋገጠነዉ፡፡ሊያስገኛቸውየሚችሉትጥቅሞችበዚህጥናትውስጥልጅዎበመሳተፉበጥሬገንዘብየሚደረግየካሳክፍያአይኖርም፡፡ የጥናቱተሳታፊዎችበምርመራዉጤትላይበመመርኮዝበሽታውበእርሶላይከተገኘለበለጠህክምናእናእንክካቤበወረዳዉጤናጣቢያመድሃኒትበነፃእንድሰጣቸዉይደረጋል፡፡

የጥናቱምስጢራዊነት

ማንኛዉምበጥናቱየተገኙመረጃዎችምስጢራዊነቱየተጠበቀነዉ ::

የጥናቱመረጃዎችበሙሉየተቀመጡትለጥናቱተብሎበሚሰጠውስውርቁጥርሲሆንጥናቱንከሚያከሄዱትባለሙያዎችበስተቀርማንምሊያ ውቅአይችልም::

የጥናቱተሳታፊማንነትበሚገልጥመልኩየተዘጋጀዉንመረጃየጥናቱተሳታፊበፊርማዉየተረጋገጠፍቃድሳይሰጥይፋአይደርግም ::

ይህጥናትሳይንሳዊመረጃእንደመሆኑመጠንበወረቀትታትሞቢወጣወይንምበሚድያቢነገርየጥናቱተሳታፊስምበምንምመልኩአይጠቀስም፡ :

ያለመቀበልወይምጥሎየመውጣትመብት

በዚህጥናትዉስጥየሚኖርዎትተሳትፎሙሉበሙሉፈቃደኝነትላይየተመሰረተሲሆንበማንኛውምጊዜይህንንጥናትየማቋረጥመብታቸዉሙ ሉበሙሉየተጠበቀነዉ::

በጥናቱባለመሳተፋቸዉወይምከጥናትበመገለላቸዉምክንያትበአሁኑወይምየወደፊትየህክምናእርዳታላይተፅዕኖአይኖርም::

የተመራማሪመገኛ

በጥናቱላይጥያቄወይምአሰተያየትካሎትየጥናቱንባለቢትአሸናፊደመቀንበዚህአድራሻማግኘትይቸላሉ::

አሸናፊደመቀ፤የድርጅቱስም - ጅማዩኒቨርስቲ /የህክምናላብራቶሪሳይንስት /ቤትተማሪ

ስልክቁጥር፡ 09-17-00-41-80

09-34-45-34-24

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ፕ.ርድልነሳውየወሀላው(0917804352)

አቶአብዲሳብሩክሰው(0911964174)

ጅማ፣ኢትዮጵያ

በጥናቱሰለተሳ ተ ፉከ ል ብአ መ ሰ ግ ና ለ ሁ ::

ANNEX II: Consent and Accent form

Part IIA: Consent form English Version

I _____, here by giving my consent for me or my child to participate in the mentioned study. I understand that this study will be used to evaluate the diagnostic performances of *NxTek™ Eliminate Malaria P.f Ag test* for the detection of asymptomatic *P. falciparum* malaria against qPCR among School children at Gomma District. I also trust that at the end of study, the results will be shared with the concerned body, Jimma university institute of health, Jimmazone health office and to the school participated.

Your child's name _____ Signature _____ Date _____

Parent's name _____ Signature _____ Date _____

Name of data collector _____ signature _____Date _____

Thank you for your participation!

Part IIB: Assent form English version for child (<18 years of study participant)

You are being asked to give a blood sample that will be examined for malaria parasite. You do not have to do this if you do not want to do, but there is no danger in doing so.

Do you agree to give your blood sample for malaria parasite examination?

Yes---------- No----------

Do you agree to take medication given to according to your result?

Yes---------- No----------

Child's name---------- signature----------Date ----------

Name of the person obtaining assent----------Signature ---------- Date _____

Witness name ---------- Signature---------- Date____

Part IIC: Assent form Amharic version

<u>የፍቃደኝነ ትመግለጫቅፅ</u>

እኔ ________________________________

በልጅት/ቤትዉስጥበሚደረገ ዉይይየ ወባበሽታመመርመሪያመሳሪያአቅምንየ መመርመርአቅምስለማጥናትበተነ በበልኝመሰረት ተረድቻለሁበተጨማሪም የምርምርዉጤቱለጅማዩኒቨርሲቲየ ህክምናሳይንስኢኒስቲቲዉትለጅማዞንጤናቢሮእንዲሁምበሚዩብ ትት/ቤትየ ሚሰራጭመሆኑልጄየ ወባበሽታበምርመራዉጤቱከተገኘበትየ ሚሰጠዉንመድሃኒትእንዲወስድፍቃደኛመሆኔንበፊር ማዬአረጋግጣለሁ

የቤተሰብስም ______________________ ፊርማ________________ ቀን _________

የእማኝስም ______________________ ፊርማ ________________ ቀን __________

የተማሪዉስም _________________ ፊርማ _________________ ቀን _________

<u>የስምምነ ትቅፅ</u>

የ ወባበሽታመመርመሪያመሳሪያአቅምንየ መመርመርአቅምስለማጥናትእየ ሰራነ ነዉ የ ደምምርመራለማድረግትፈልጋለክምርመራ ወምንምአይነ ትቼግርእናጉዳትአያስከትልብክምስጋትየ ምትሰራዉእየ ደለምካልፈለክግዳጅየ ለወም ገ ርግንበምርምሩዉጤ ትበሽታዉከተገ ኘበትየ ሚሰጠዉንመድሃኒ ትበነ ፃ እናቀርብልካለንምርመራለማድረግፍቃደኛነ ክ?

አዎ ____________ አይደለሁም____________

በዉጤቱከመሰረትመድኃኒ ትለመዉሰደፍቃደኛነ ክአዎ ______ አይደለሁም_______

የተማሪስም ______________ _______ ፊርማ_____________ ቀን ______________

ስምምነ ትያስፈፀመዉም _____________ ፊርማ _____________ ቀን ___________

የእማኝስም ______________________ ፊርማ _____________ ቀን ___________

Part IID: Assent form Afaan Oromo version

<u>GucaFedhiiibsuu</u>(Hirmatotaumirii waggaa18 gadita'aniif)

Aniobboo/Addee _____

qorrannooJiraattotanaannooZooniiJimmaaaanaaGommaaworredaairrattidandeettiimeeshaadhukkubabus aayaaluufgargaarumadaaluuBu'aanqorannochaaqaamailaaluuhundaaf,

DhaabataFayyaaUniversitiiJimmaa,

WaajiraEguumsaFayyaazooniiJimmaatiifakkasumasmanabaruumsichaatiifakkadhihaatuuniamanama.

MaqaaMaatii _____ Mallattoo _____ Guyyaa _____

MaqaaRagaa _____ Mallattoo _____ Guyyaa _____

MaqaaQorataa _____Mallattoo _____ Guyyaa_____

Gucawaligaltee(Hirmatotaumirii waggaa18 gadita'aniif)

AmmakansigaafachaajirrusaamudadhiigaaqorannoodhukkubaJiraattotanaannooZooniiJimmaaaanaaGo mmaaworredaairrattidandeettiimeeshaadhukkubabusaayaaluufgargaarumadaaluu nu gargaaruakka nu kenituuf. Kennuskennuudhisuusnidandeessagaruukeennuukeetiinwanttimidhamtutokkoilleenhinjiru.

Saamudadhiiga nu kennuufwaligalteerta?Eeyyee_____ lakki_____

Eeyyee _____ lakki _____

MaqaaDa'imaa _____ Mallattoo _____Guyyaa _____

MaqaaNamaGaafatee _____ Mallattoo_____ Guyyaa _____

MaqaaRagaa _____ Mallattoo _____ Guyyaa_____

Galatomaa!!!

ANNEX III: procedure for capillary blood sample collection

Materials

1. Disposable gloves

2. Automatic lancets
3. Alcohol (70%) and gauze
4. Clean microscope slides
5. Capillary blood sample
6. Pencil
7. Gloves
8. Waste and sharps disposal containers

Procedure

1. Label the slide with the student's unique code
2. Assemble the necessary materials and equipment
3. Identify the student
4. Select the finger either from the left or right hand
5. Prepare and clean the finger by swab using a cotton ball saturated with 70% alcohol
6. Allow the site to dry
7. Penetrating the finger with a sterile lancet and taking the blood
8. Place a small drop of blood 1.0 cm far from the frosted end of the slide
9. The spreading slide is placed in front of the drop of blood at an angle of about 30^{o}-40^{o} to the slide and then is moved back to make contact with the drop
10. The drop will spread out quickly along the line of contact with the slide
11. The spreader is advanced with a smooth steady motion so that a thin film of blood is spread over the slide
12. Allow the smear to air-dry

ANNEX IV: procedure for preparation of Giemsa stock and working solution

Preparation of Giemsa (0.76%) stock solution

To our study when we use the solution from the market the staining is not good enough and it doesn't allow for observing the platelets, cytoplasm of WBC, and RBC. So, we decided to prepare the stock solution from the powder by following WHO protocol (54). After preparing the stock solution, 10% Giemsa working solution is prepared. The solution was made daily that works only for 8 hours a day. To ensure the quality of the prepared working solution, we got a better staining solution when compared with Giemsa working solution available on the market. Finally, the slide contains visible platelets, the cytoplasm of the white blood cells, and the red blood cell.

Supplies and Equipment

1. 3.8gm Giemsa powder
2. 250ml Glycerol
3. 250ml Methanol
4. Whatman filter paper
5. Measuring cylinders
6. Pasture pipettes
7. Containers
8. buffered water, pH 7.2
9. beaker or tube

Procedure for Giemsa stock solution preparation

1. weighed 3.8 Giemsa powder on a digital balance
2. Add 250ml absolute methanol
3. Add 250ml glycerin (after adding those materials shaking frequently)
4. Incubate at 60ºC incubator for one week with frequent shaking
5. Filtered by Whatman filter paper

Procedure for 10% Giemsa working solution preparation

1. Place 9 mL of distilled water into a beaker or tube
2. Filter the Giemsa stock solution through filter paper and transfer it to a 25 to 50 mL container
3. Add 1 mL of Giemsa stock solution
4. Use stain within 15 min of preparation, and discard unused stain

Giemsa staining and examination

Procedure

1. Place the air-dried smear film side up on a staining rack
2. Cover the smear with Giemsa stain and leave for 10 minute
3. Place the Giemsa stained smear film side up on a staining rack
4. Wait until the stained smear is dried
5. Add a drop of oil immersion on the slide and
6. Examine under 100x magnification power

ANNEX V: The general instruction of NxTek™ Eliminate Malaria P.f Test

1. Check the expiration date. If the device is expired use another kit
2. Put on the glove, use the new glove for each patient
3. Open the test pouch and write the patients name or code on the test device
4. Open the alcohol swab and clean the patient's 4^{th} finger. The alcohol must be dried before pricking or the test may not work
5. Pick the patient's finger with the sterile lancet. Wipe away the first drop of blood much sterile gauze or cotton
6. Use the inverted cup to collect the drop of blood
7. Add drowned specimen of blood into the round specimen well
8. Add 4 drops of assay diluent into the square assay diluent well
9. The control line should appear for all results. If it doesn't appear the result is considered invalid and the specimen should be refused using a new test kit.
10. Read the test result after 20 minutes. Do not read test results after 30 minutes. Which results from erroneous results

ANNEX VI: DNA extraction procedures

Material and reagents

1. Micro-centrifuge (>13000 rpm)

2. Water bath (95ºC)
3. Double distilled water (ddH_2O)
4. Eppendorf tubes (1.5 ml)
5. Falcon tubes (conical ended of 50 ml)
6. ¼ round hole puncher (manufactured in China, Catalogue no. BSN62895)
7. 10% saponin (FlukaBiochemika, Catalogue number: 47036, Sigma-Aldrich chemieGmbh, Riedstr. 2, D-89555 Steinheim, 07329/970, Germany)

❖ **Preparation** (10% saponin): 1.0g of saponin powder in 10 ml of double-distilled water in a falcon tube

8. 1*phosphate buffered saline, PBS (Catalogue number. 198886, expiry date 06/2022. Fisher Scientific. JassenPharmaceuticalaan, 2440 Geel-Belgium)

❖ **Preparation**: Dissolve one tablet in 200 ml ddH_2O

9. 20% Chelex-100 (Sigma, Cat no. p44417, Bio-Rad, #412822)

❖ **Preparation** (20% Chelex-100 solution): Fill the Chelex up to 15 ml of falcon tube and Add ddH_2O up to 40 ml of the falcon tube

PROCEDURES

1. Use the round hole puncher to cut approximately 3-5 mm pieces of the Dried Blood Blotted (DBS) filter paper and put them into 1.5 ml labeled Eppendorf tubes and clean the puncher after every sample
2. Add 950 µl 1*PBS and 50 µl 10% saponin in the Eppendorf tubes containing the sample
3. Mix well in the thermomixer, and then incubate for 4ºC for > 4 hours or overnight
4. Centrifuge the mixture at 14000 rpm for 10 minutes at room temperature and discard the liquid content
5. Remove(wash) any remnants of saponin by adding 1ml (1000 µl) of PBS and then spine at 14000 rpm for 5 minutes
6. Discard the PBS and spine the tubes for 30 seconds, and remove the liquid by 200 µl pipet
7. Dry the DNA (on the filter papers) within the Eppendorf tubes left open for 15 minutes at room temperature
8. Add 150 µl of 20% Chelex suspension and 100 µl of ddH_2O to extract the parasite DNA by incubating the mixture at 95ºC in the water bath for 10 minutes, vortex the mixture every 2 minutes in the process of inoculating and extracting
9. Centrifuge the mixture at 14000 rpm for one minute

10. Transfer the parasite DNA into sterile Nunc tubes (*Trademark and Manufacturer??)* of 96-well S-plate or 0.5 ml tubes
11. Keep the DNA at -20 ºC for PCR

ANNEX VII: Detection of *plasmodium* parasite species

TaqMan probe detection of plasmodium parasite Species-Specific detection

Material and Reagents

1. Primer and Probes for both P. falciparum and P. vivax
2. qPCR master mix (PerfeCTa® qPCR ToughMix®, Low ROX ™, Quanta Bio vwr catalogue number: - 97065-968, Cost: $2485.19 for 50 ml)
3. PCR plate (Fisher Scientific catalogue number AB1400150. Cost: $284.87/case_150)
4. qPCR machine (QuantStudio3. Applied Bio system Multiplex Real-time PCR Instrument)

Multiplex Real-time quantitative (qPCR), QuantStudio3

Note:the standard molecular strength of each primer is 10 µM, and a probe is 2 µM.

1. Prepare the following master mix in a 1.5 ml tube (Eppendorf tube)

a) Preparation formula for primer working solution:
   - ❖ Add 50 µl of each primer stock solution into 450 µl molecular grade water

b) Preparation formula for probe working solution:
   - ❖ Add 10 µl of the probe stock into 490 µl molecular grade water (ultrapure water that is certified RNase, DNase, and Protease free**)**

A. Before starting wrap the tube in aluminum foil to protect sunlight.
B. After thawing, keep the qPCR Master Mix in the fridge if used frequently and store in a deep freezer that is not used for > 2 weeks.
C. Avoid frequent freeze-thaw of the probe. Once thawed, keep in the fridge for up to a month

2. Multiplex detection of Pf and Pv

Master Mix	1x(µl) - for one PCR well	100x(µl)- final volume

PerfaCTa(2x)	6.0	600
Pf-FAM (probe)	0.5	50
Pv-VIC (probe)	05	50
F-F(Pf- forward primer)	0.4	40
F-R (Pf- Reverse primer)	0.4	40
Pv-1 (Pv- forward primer)	0.4	400
Pv-2 (Pv- Reverse primer)	0.4	400
DNA	2.0 µl	-
ddH_2O	1.4	140
Total volume	**12**	**1000**

3. **Seal the plate with optical PCR foil**
    a) Use the plastic tool or fingers covered with tissue to press on the foil, in particular at the sides and corners after inserting it into the Quant Studio qPCR machine
    b) Centrifuge (use an empty plate for balance or mix using vortex for 30 seconds
4. **qPCR program running**
    a) Label wells reading: - Probe Pf as FAM, Pv as VIC
    b) Label Samples well and standard wells
    c) Check the cycling conditions
5. **Total running time**
    a) Confirm the total running time = one hour (60 minutes)
    b) In the "Run" tab (computer connected-machine),
        i. select the instrument
        ii. press starts
        iii. Save you experiment

DECLARATION

I, the undersigned, declare that this thesis is my original work, has not been presented for a degree in this or any other university, and that all sources of materials used for the thesis have been fully acknowledged.

Name: Ashenafi Demeke
Signature: _____
Name of the institution:Jimma University
Date of submission: 16/05/2022

This thesis has been submitted for examination with my approval as University advisor

Name and Signature of the first advisor _____

Prof. Delenasaw Yewhalaw (Ph.D.)

Name and Signature of the second advisor _____

Mr. Abdissa Biruksew (MSc, Ph.D. fellow)

Examiner 1: Dr. Lemu Golasa _____ _____

Examiner 2: Dr. Teferi Eshetu _____ _____