FIELD EVALUATION OF A NOVEL ONE STEP MALARIA *P.F* AND *P.F/P.V* RAPID DIAGNOSTIC TESTS IN PWANI REGION, TANZANIA

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ABSTRACT

Malaria rapid diagnostic tests (mRDTs) play an important role in the early detection of symptomatic and asymptomatic malaria in endemic areas. While several mRDTs are currently on the market, the availability of mRDTs with high sensitivity and specificity will add value in the fight against malaria. We evaluated the field performance for malarial diagnosis of a novel One Step Malaria (*P.f/P.v*) Tri-line and One Step Malaria *(P.f*) rapid test kits in Pwani, Tanzania. In a cross-sectional study conducted in Bagamoyo and Kibiti districts in Tanzania, symptomatic patients were tested using the SD BIOLINE, One Step Malaria (*P.f/P.v)* Tri-line and One Step Malaria *(P.f)* rapid test kits, microscope and qPCR. Samples tested positive by microscope, and verified by qPCR as the confirmatory method were analysed to determine sensitivity of the test kits. An additional qPCR assay was carried out to detect HRP-2 gene deletion on mRDT negative but microscope and qPCR positive samples. The sensitivity and specificity of One-step *P.f/P.v* Tri-line mRDTs was 96.0% (CI: 93.5% to 97.7%) and 98.3% (CI: 96.8% to 99.2%) respectively. One step *P.f* mRDT had sensitivity and specificity of 95.2% (CI: 92.5% to 97.1%) and 97.9% (CI: 96.3% to 99.0%), respectively. Positive predictive value (PPV) was 97.6 % (CI:95.4% to 98.7%) and negative predictive value (NPV) was 96.2% (CI:95.5% to 98.3%) for the One-step *P.f/P.v* Tri-line mRDTs., while One step P.f mRDT had positive predictive value (PPV) and negative predictive value (NPV) of 97.0% (CI:94.8% to 98.3%) and 96.7(CI:94.9% to 97.9%), respectively. 9.8 % (CI: 7.84 -11.76) of all samples tested and reported to be malaria-negative by mRDT had HRP-2 gene deletion. One Step Malaria *(P.f/P.v)* Tri-line and One Step Malaria *(P.f)* rapid test kits have similar sensitivity and specificity as the standard mRDT that is currently in the market, demonstrating the potential to contribute in the fight against malaria in endemic areas. However, the identified malaria parasites population with HRP-2 gene deletion pose threat to the current mRDT usefulness in the field and warrants further investigations.

DECLARATION

I, Zena E. Mwangonela do hereby declare to the Senate of the Nelson Mandela African Institution of Science and Technology that this dissertation titled "*Field Evaluation of a Novel One Step Malaria P.F and P.F/P.V Rapid Diagnostic Tests in Pwani Region, Tanzania*" is my original work and has never been or intending to be submitted for a degree award in any other institution.

Zena E. Mwangonela Date

Dr. Grace Mwangoka Date

Dr. Esther G. Kimaro Date

Dr. Clement Mweya Date

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CERTIFICATION

The undersigned certify that they have read and hereby recommend for acceptance by the Senate of the Nelson Mandela African Institution of Science and Technology a thesis titled "Field Evaluation of a Novel One Step Malaria P.F and P.F/P.V Rapid Diagnostic Tests in Pwani Region, Tanzania" in partial Fulfilment of the Requirements for the Degree of Master of Science in Public Health Research of the Nelson Mandela African Institution of Science and Technology.

Dr. Grace Mwangoka Date

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DEDICATION

I would like to dedicate to my husband, who was behind me for every step, encouraging, supporting and advising me throughout the study. My relatives for best wishes, my fellow student MSc-PHR IHI 2021 for positive interaction and endless support.

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LIST OF ABBREVIATIONS AND SYMBOLS

BS	Blood Smear
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
HRP-2	Histidine-rich Protein 2
IHI	Ifakara Health Institute
MRDTs	Malaria Rapid Diagnostic Tests
NA	Non Applicable
NIBSC	National Institute for Biological Standards and Control
NPV	Negative Predictive Value
P.f	Plasmodium Falciparum
PPV	Positive Predictive Value
P.v	Plasmodium Vivax
qPCR	Quantitative Polymerase Chain Reaction
WHO	World Health Organization
ITN	Insecticide-Treated Nets
TMIS	Tanzania Malaria Indicator Survey
LLTNs	Long Lasting Treated Nets
SOP	Standard Operating Procedure

CHAPTER ONE

INTRODUCTION

1.1 Background of the Problem

Malaria remains an important public health concern and a serious vector-borne disease worldwide (Wang & Mihayo, 2021). It continues to be a major public health concern in the endemic areas despite enormous effort and funding invested in the fight against malaria to date (Tizifa *et al.*, 2018). According to the WHO malaria report of 2022, malaria deaths decreased by the period of 2000 to 2019. The report showed that there were 897 000 deaths in 2000 and decreased to 577 000 in 2015 down to 568 000 in 2019. However, between 2019 and 2021 during COVID-19 pandemic disruption of health care services which resulted in a reduction in resources for malaria control. Interventions measure such as lockdown and travel restriction imposed during pandemic disrupted malaria control programs such as indoor residual spraying, bed net distribution and mass drug administration which led to 63 000 malaria deaths (*World Malaria Report,* 2022).

In Africa, the report shows that many countries affected by malaria and among them include Nigeria (31%), Democratic Republic of Congo (13%), Niger (4%) and the United of Tanzania (4%). To reach zero malaria, more efforts and control, especially in Sub-Saharan countries is needed (Diouf *et al.*, 2020; Badmos *et al.*, 2021) Tanzania reported to account for over half of all malaria deaths that occurred globally (WHO, 2021). These statistics could be attributed to failure to reach the 2020 target for insecticide-treated nets (ITN) distribution, challenges associated with increasing prevalence of asymptomatic cases that could not be detected by routine diagnostic methods, or due to interruption of health care systems during COVID-19 pandemic (Bylicka-Szczepanowska & Korzeniewski, 2022). Despite disruptions during the COVID-19 pandemic there is progress towards elimination and reduction of the malaria burden.

To support malaria elimination campaigns in Tanzania, different malaria vector control interventions have been deployed over the years including provision of long-lasting insecticidal nets (LLINs), indoor residual spraying (IRS) and by targeting the breeding sites through applying the insecticides (Wangdi *et al.*, 2018). However, despite these interventions the morbidity and mortality persisted to under-five children and pregnant women (Imboumy-Limoukou *et al.*, 2020).

Diagnostic tools are however among the important pillars of elimination strategies as it is key for clinical management of the disease. When malaria parasite detection is done early, it facilitates early management that would prevent progression to severe disease and reduces the risk of transmitting the infection to the next person. Routinely, microscope and rapid diagnostic tests (RDTs) are widely used for parasite detection in both rural and urban settings. Nevertheless, each

technique has its strength and weakness in terms of performance sensitivity and specificity. Malaria RDT (mRDT) is a diagnostic method that can detect parasite antigens in the whole blood samples. When compared to other diagnostic techniques, it is simple to run, provides results in a short time and does not require electricity or expensive equipment (Justyna, 2017). While several mRDTs on the current market are showing sub-optimal performance in the field (D'Acremont *et al.,* 2009) the most reliable mRDTs will be the one that can improve detection to enable effective treatment of confirmed cases to reduce complications related to severe forms of malaria. The most sensitive mRDTs that are currently in the market detect parasite histidine-rich protein 2 (HRP-2) circulating in the blood. These proteins are specific to *P. falciparum*, and expressed in abundance by asexual parasites (Ogunfowokan *et al.,* 2020).

Diagnostic testing as an effective intervention package for malaria control and elimination strategies (WHO, 2018), led to many malaria endemic countries to adopt the World Health Organization policy of testing suspected malaria patients by rapid diagnostic test (RDT) before antimalarial treatment initiation (Harvey *et al.,* 2008; Zurovac *et al.,* 2008). In 2017, National Malaria Programs distributed 245 million RDTs. In WHO African Region with 92% contribution of malaria cases; RDT is the most preferable test method in all levels of health facilities as 75% of malaria tests were conducted using RDT (WHO, 2018). In 2010, Tanzania introduced RDTs in some regions and later deployed throughout the country. The high coverage of RDTs in the country has managed health facilities to provide quality malaria case management (Gürel, 2011). In addition, the 2017 Tanzania Malaria Indicator Survey (TMIS) in collaboration with other stakeholders indicated a decline of malaria prevalence up to 7.3% in children aged 6-59 months with positive RDT results (NBS, 2018). Therefore, high quality of RDT is very crucial to ensure that the milestones for 2030 are reached (NBS, 2018).

Errors in malaria rapid diagnostic tests include giving false positive and false negative results may occur. Many factors can results in errors such as: human errors this is especially when the test is not performed according to manufacturer's instructions or if the person performing the test is not properly trained (Watson *et al.,* 2019). The test cannot provide good results if it is not working properly or test kits are expired. Additionally, the test may not be sensitive enough in areas where the malaria parasite load is very low. All these errors can lead to misdiagnosis and inaccurate results which can have serious consequences for patients including delays in treatment and complications and even death can occur (Berhane *et al.,* 2017).

There are measures that can minimize errors of RDTs performance such as careful selection process (World Health Organization, 2011), proper storage conditions of the tests, adequate labelling, clear performance instructions, and ongoing training laboratory technicians (Harvey *et*

al., 2008). Proper knowledge should be provided to personnel to avoid misdiagnosis which can lead to patients being treated for malaria when they don't have it, or not treated when they do. Also when knowledge is provided, it will avoid delays in getting accurate results and interpretation. In large-scale implementation of screening programmes based on RDTs, the establishment of a quality assurance (QA) unit is required.

In 2017 WHO provided a guideline titled "TSS-3 Technical Specifications Series for submission to WHO Prequalification-Diagnostic Assessment: Malaria rapid diagnostic tests". The major objective for the guideline is to give a standard technical guidance for assessment of in vitro diagnostic medical devices (IVDs) to the manufacturers who are seeking WHO prequalification of IVDs, RDTs for malaria diagnosis (Global Malaria Programme, 2017). The manufacturers shall submit evidence of the clinical performance of the intended devices showing the procedures used to ensure the devices have been correctly operated by the users, detected the target pathogens and its full indication for use. The clinical performance studies for the malaria rapid diagnostic tests (mRDTs) must be conducted in low- and middle-income countries, where the devices are likely to be used by a diversity of knowledge and skills. It should also cut across the population such as paediatrics, symptomatic patients and those with clinical indication.

Ifakara Health Institute (IHI) was one of the selected site to conduct prequalification assessment for two mRDTs products, the One Step Malaria *P.f/P.v* triple line and One Step Malaria *P.f* mRDTs manufactured by InTec (PRODUCTS, INC.). The One Step Malaria *P.f/P.v* Tri-line Test is a colloidal gold enhanced, two-site sandwich immunoassay utilizing whole blood for the detection of *P. falciparum* specific histidine rich protein-II (P.f HRP-II) and *P. vivax* malaria specific parasite lactate dehydrogenase (pLDH). This rapid test aids in the diagnosis of relative infection (a proportion of individuals in a population who have been infected with malaria) and relative symptoms which are commonly associated with the disease by providing preliminary analysis results. The obtained results are analysed in connection with other information, e.g. clinical symptoms, and alternate tests to make final decision.

A monoclonal antibody against *P.f* HRP-II and *P. vivax* malaria specific pLDH are immobilized in the test region on nitrocellulose membrane. The red blood cells are lysed releasing *P.f* HRP-II and *P. vivax* specific pLDH, which bind selectively to this monoclonal antibody and migrate chromatographically on the membrane by the capillary action. The signal reagent coated with specific antibodies will bind with the antibody-antigen complex, producing a purplish line. To ensure assay validity, a purplish red control band in the control region will appear regardless of the test result. Only when the control band appears the assay is valid.

1.2 Statement of the Problem

Rapid and accurate diagnosis of malaria parasites is crucial for the reduction of morbidity and mortality, drug resistance and proper patient management as well as implementation of suitable control strategies (Berzosa *et al.,* 2018). Despite the remarkable benefits of the mRDTs, batch-to-batch variations, result misinterpretation, limited sensitivity and specificity of some test kits, and instability of stored test kits in tropical climate offers some disadvantages. Good performance of mRDTs depends on how sensitive and specific they are (Galatas *et al.,* 2020).

Moreover, previous studies conducted in the Amazon region identified patients infected with *P. falciparum* strains that had acquired deletions in the genes that encode these proteins (P.fHRP-2 and P.fhrp3), rendering them undetectable by HRP-2-based RDTs (*Fontecha et al*., 2018; *Leonard et al*., 2022). Since then, many studies have demonstrated the presence of such mutated strains in other countries and regions including Tanzania (Kaaya *et al.,* 2022). The frequency and global distribution of the HRP-2 gene deleted parasites population is not yet fully understood, although, the relative incidence of these deletion mutants has been reported to threaten the usefulness of HRP-2-based RDTs (Berzosa *et al.,* 2020; Parr *et al.,* 2017). Screening for HRP-2 gene deleted parasites during the field evaluation of mRDTs is critical to inform the public health official on the threat and proposing improved methods for malaria diagnosis in the affected population.

An addition of new mRDT with improved performance sensitivity and specificity from the current battle against malaria will supplement the existing RDTs when there is scarcity especially in remote areas where there is still inadequate number of tools. The InTec PRODUCT INC Company from China introduced new mRDT kits for detecting the *Plasmodium falciparum* and other Plasmodium species. However, the field performance evaluation of these kits in terms of sensitivity and specificity is needed. The study aimed to evaluate the field performance sensitivity and specificity of the new One Step Malaria (*P.f/P.v*) Tri-line and One Step Malaria *(P.f)* rapid test kits by comparing with microscopy and qPCR as reference method.

1.3 Rationale of the Study

Most malaria RDTs have been used to detect malaria species but have the limitation of detecting a low concentration of parasites in areas with low malaria transmission. The RDTs performance in terms of sensitivity and specificity deteriorate where the ability of the test to detect accurately the presence or absence of malaria antigen in the blood sample become limited. The goal of malaria-endemic countries, including Tanzania, is to achieve elimination by 2030. To achieve this, timely detection and treatment of all cases is critical, otherwise, transmission will inevitably persist. Thus highly sensitive tools-contribute to acceleration toward malaria elimination by

supporting research and development on new tools and strategies for malaria prevention, diagnosis and treatment. As for the new brands of malaria RDTs, it is essential that their field performance is evaluated. The importance of this study was to see the value addition to overcoming limitations from those which are in use by evaluating the new mRDTs performance sensitivity at different age groups and malaria transmission intensity. Better evaluated mRDTs will improve rapid case detection, effective treatment of confirmed malaria cases and help to avoid long-term complications related to severe forms of malaria and development of resistant strains, which might result from incorrect diagnosis and irrational use of drugs. Also, the results from this study will allow InTec PRODUCTS, INC to obtain true point of care data to submit to WHO for Pre-qualification certification and later registration in the proposed market where the product is intended. Moreover, the results will also allow the company to assess if the sensitivity and specificity of the tests need to be improved in any way.

1.4 Research Objectives

1.4.1 General Objective

To evaluate the field performance of new malaria RDTs, One Step Malaria *P.f/P.v* triple line and One Step Malaria *P.f* at different malaria transmission intensity in Pwani region.

1.4.2 Specific Objectives

(i) To assess sensitivity and specificity of new, One Step Malaria *P.f/P.v* triple line and One Step Malaria *P.f* with reference to microscope and qPCR from different malaria transmission intensity.

(ii) To compare detection performance of new malaria RDT; One Step Malaria *P.f/P.v* triple line and One Step Malaria *P.f* using microscopy and qPCR as a reference method in the different malaria transmission intensity.

(iii) To assess HRP2 gene deletion for negative samples to RDTs but positive for microscope and qPCR.

1.5 Research Questions

(i) What proportion of infection is missed when using malaria rapid diagnostic tests varying in their performance accuracy in different age groups and malaria transmission areas?

(ii) How the new malaria RDTs perform accurately in terms of sensitivity and specificity when compared to the microscope and qPCR?

(iii) What is the proportion of HRP2 gene deletion in the study areas?

1.6 Significance of the Study

China has experience and lessons in malaria control and prevention programs. Currently, China is sharing experience achieved from its 1-3-7 model to malaria elimination stage by implementing it in surveillance systems through China-UK-Tanzania Malaria Pilot Project in Tanzania. Such a strong surveillance system provides the best platform for mRDTs prequalification studies. This study aim to provide evidence of the field clinical performance of the One Step Malaria (P.f/P.v) triple line and One Step Malaria (P.f) tests manufactured by InTec PRODUCTS, INC and to demonstrate that reasonable steps from manufacturing process to the end user have been well considered.

1.7 Delineation of the Study

The RDT is the most preferable test method in all levels of health facilities as 75% of malaria tests are conducted using RDT. In 2010, Tanzania introduced mRDTs in some regions and later deployed throughout the country. The high coverage of mRDTs in the country has managed health facilities to provide accurate management of malaria cases. In addition, the 2017 Tanzania Malaria Indicator Survey (TMIS) in collaboration with other stakeholders indicated a decline in malaria prevalence up to 7.3% in children aged 6-59 months with positive mRDT results. Better mRDTs performance improve case detection, effective treatment of confirmed malaria cases and help to avoid long-term complications related to severe forms of malaria and development of resistant strains, which might result from incorrect diagnosis and irrational use of drugs. This study is intended to perform a field evaluation of the new One Step Malaria (Pf/Pv) triple line and One Step Malaria (Pf) mRDTs manufactured by InTec PRODUCTS, INC. First, the current One Step Malaria P.f/P.v Tri-line and One Step Malaria P.f mRDTs test kits were designated to detect antigen for P.falciparum and P.vivax species only and no other malaria species like *P. malariae* and *P. ovale and P. knowles*. This might led to misidentification of etiology of non-falciparum and non-vivax fever. Moreover, the current study did not analyzed antibodies against P.fHRP-2 by Enzyme Linked Immunosorbent Assay (ELISA) to determine whether there was presence of HRP-2 proteins in individuals who had HRP-2 gene deletion. Although, the qPCR itself is sufficient to confirm gene deletion, the results from antibodies could have been a positive supplement for the finding. In additional, the detection of other co-infection was sorely depended on clinical judgments made by clinicians using national treatment algorithms and not laboratory testing. Lastly, the cost effectiveness of the new test kits were not been established during this study hence advise the manufacturer to sell the kits in affordable price.

CHAPTER TWO

LITERATURE REVIEW

2.1 Malaria Pathogenesis

Malaria is a vector-borne disease caused by a parasite of the genus Plasmodium (Dejazmach et *al.,* 2021). *Plasmodium falciparum, Plasmodium malariae, Plasmodium ovale Plasmodium vivax and Plasmodium knowlesi* are malaria parasite species which are responsible for transmission of malaria in humans. These species undergo different morphological changes from simple to complex cell replication which later lead to disease after infecting human cells (Milner, 2018).

Malaria parasites goes through a complex life cycle of asexual replication and sexual stages development. A parasite sporozoites enters the bloodstream through the bite of an infected mosquito and moves to the liver stage where it begins to invade hepatocytes. In the liver the sporozoites differentiate to form merozoites. The matured merozoites are released into the blood stream and invade red blood cells leading them to rupture and realize more merozoites to invade new RBCs (Jensen *et al.,* 2020).

Small merozoites develop into gametocytes which develop into sexual forms which then mate in the mosquito midgut and produce new sporozoites that migrate into the salivary grand and can infect a human host through mosquito bite. During the cell rupture, the parasite is responsible for the symptoms of malaria (Amoah *et al.,* 2020). Figure 1 shows different stages where the parasite is infecting human cells.

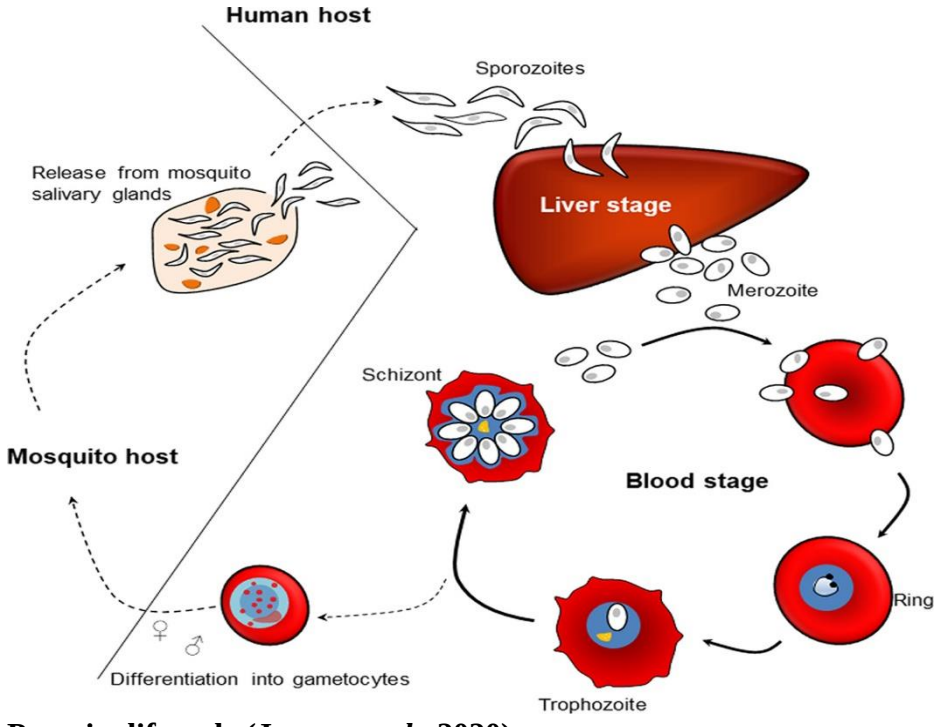

Figure 1: Parasite lifecycle (Jensen *et al.,* 2020)

Globally, the burden of morbidity and mortality is pushing more efforts in prevention, diagnosis as wells as treatment (Juki & Vˇ, 2019). Due to increase of deaths from malaria various efforts are being made to ensure prevention and spread.

2.2 What Interventions have been Done?

To support the malaria elimination campaign, different malaria vector control interventions have been done for years. Achievements to reduce the burden of malaria transmission contributed by vector control programs through targeting the breeding sites by applying the larvicides. Among the interventions includes the provision of insecticide-treated nets (ITN) and indoor residual spraying (IRS) which are used to spray the walls of the houses (Wangdi *et al.,* 2018). The ITNs has deigned to repel or to kill mosquitos that come into contact with it. The ITNs have advantages in killing mosquitos but they last for six months so they need to be retreated several times after every six months. To overcome the weaknesses of ITNs, long lasting treated nets (LLTNs) were introduced. These were considered to be an improvement over ITNs because they provide longer lasting protection (three years) and require less maintenance however both types of nets are effective in reducing malaria when used consistently (Zerdo *et al.,* 2020).

Moreover, intervention is done through management of larva sites (where mosquitoes lay their eggs) to prevent the development of the mosquitoes larvae into adult mosquitoes. This is done through environmental management by destroying the bleeding sites like removing standing water, and cleaning debris. Also, natural predators such as fish or bacteria can be used to control mosquito population (Walker & Lynch, 2007). However, negative consequences may occur as risks to humans if not used effectively for example killing non-targeted organisms, contaminating water sources and posing health risks especially for those who come into direct contact with chemicals.

Fighting against malaria should be contributed by government policy and social behaviour campaigns by making strategies that aim to reduce the incidence of malaria by providing insecticide treated bed nets, indoor residual spraying and antimalarial drugs. Investment in conducting research to improve the understanding of malaria transmission, and drug resistance should as well be considered by making sure the drug is available and accessible. Provision of education on how to maintain our environment and identifying areas for improvement (Nalinya *et al.,* 2022) and insuring community engagement, effective treatment, evaluating malaria control programs and improvement of housing for the majority of people to avoid unnecessary malaria morbidity and mortality rates (Finda *et al.,* 2020) are major supplement for malaria intervention program.

Apart from the success made in the malaria control and elimination program, challenges remain for diagnostic tools for clinical management of the disease despite playing a big role in the malaria control program (Santos *et al.,* 2020).

2.3 Malaria Diagnosis Tools

Diagnosis tools are essential in eradication, control, and management of malaria cases. Rapid and accurate diagnosis of malaria parasites is crucial for proper patient management as well as implementation of suitable control strategies (Berzosa *et al.,* 2018). Rapid diagnostic tests (RDTs, and Microscopes are the most common diagnostic tools that are used in most health care facilities and Polymerase Chain Reaction (PCR) is mostly for research purposes. Assessing the performance of the diagnostic tool is essential for reliable results (Adebisi *et al.,* 2018).

The PCR is a highly sensitive technique since it can detect a low concentration of parasites in the blood sample with low parasitemia (Britton *et al.,* 2016). It is able to detecting RNA or DNA of the malaria parasite in the blood samples. The PCR is useful for detect low level infections or confirming the presence of the parasite in cases where microscopy or RDTs are inconclusive. PCR can detect small amount of RNA or DNA through amplification of the specific region, 18S rRNA gene (Kamau *et al.,* 2011) and making more copies of DNA or RNA making it easier to identify the infection. It undergoes three steps including denaturation, annealing and extension (Rahi *et al.,* 2022). Denaturation involves heating the DNA sample to a high temperature which is usually 95℃ to denature it and separate s the double-stranded DNA into single strands. Annealing (50-60℃) is when the temperature is lowered to allow primers to bind to the single DNA and sequences which are complementary to specific DNA to be amplified. Extension is usually around 72℃ the DNA polymerase enzyme extends the primers by adding nucleotides along the template strand creating new double strand DNA (Sazed *et al.,* 2021). Regardless of the benefits of this technique, this technique is not used for routine diagnosis because it is more expensive based on the equipment, reagents, electricity and need highly qualified personnel to run and interpret the results.

For decades, microscopy has been the gold standard for malaria diagnosis in health care facilities due to its advantage of accuracy in the identification of parasites to the species level (Muhammad *et al.,* 2023). Microscopic parasites are identified by two techniques, thick and thin smear. Thick smear involves concentrating the malaria parasite in a small area which makes it easier to detect their presence. It is involves placing a drop of blood sample on a slide then spread it thickly over a large area and allowed to air dry then stained with suitable dye such as Giemsa stain (Bejon *et al.,* 2006). While in thin smear, a drop of blood is spread thinly on a slide allowed to air dry and stained by Giemsa stain so as to allow a better visualization of malaria parasite morphology. The

thin smear is mostly used for speciation (plasmodium species) of the parasite and to determine the percentage of infected red blood cells (WHO, 2016). There are several plasmodium species, including *Plasmodium falciparum* which is responsible for malaria infection and can be fatal if left untreated. It is identified by the presence of ring shaped in the red blood cells. *Plasmodium vivax* which is identified by the presence of enlarged red blood cells but *Plasmodium malariae* which form a malaria that can last for several years is identified by presence of small ring- shaped in a red blood cells. Also there is *Plasmodium ovale* which is identified by the presence of oval shaped parasites in the red blood cells. Generally Plasmodium species can be identified by their size, shape ,and appearance of their parasites in the red blood cells (Kho *et al.*, 2022). However, microscopy requires laboratories equipped with infrastructure such as electricity, skilled personnel and expensive instruments which are limited in most African settings (Falade *et al.*, 2016).

Though microscopy is used as the gold standard and PCR as the most sensitive technique, WHO approved the use of antibody-based Rapid diagnostic tests (RDTs) that detect malaria antigen in a blood sample. The RDT is currently the most widespread method for malaria diagnosis in developing countries (WHO, 2009). Malaria rapid diagnostic tests detect the presence of malaria parasite antigen in human blood. It detects proteins produced by malaria parasites in the blood of infected individuals. They use vertical flow, lateral flow and flow- through for detection of these proteins.

Vertical flow is when the test strip allows vertical flow of sample and reagents. The sample is applied to the top of the test strip and flows down through the membrane. The reagent in the strip reacts with the protein producing a visible line on the strip if a malaria parasite is present. It is designed to allow sample and detection reagent to flow through the membrane by capillary action. The sample is applied to the sample pad and the detection reagents are applied to the conjugate pad. As the sample flow through the membrane it encounters the detection reagent which bind to the target antigen if it is present. The bound detection reagent complex flow further down the membrane and is captured by capture reagent. The captured complex produces a visible colored band which used to determine the concentration of antigen in the sample (Rogier *et al.*, 2017). Lateral flow is when the sample is applied to the end of the strip and flows laterally through the membrane and reagent reacts with the protein in the sample producing visible line on the strip if malaria parasite is present. Typically it is including sample pad where the sample is applied, conjugate pad as the labelled antibody where the antigen bind, membrane the area where the antigen-antibody complex captured, absorbent pad where the sample and conjugate absorbed, test line where antigen-antibody complex is captured and detected and the last is control line where the control antibody is captured and detected (Amplification *et al.*, 2020).

While flow-through is when the sample is applied to the sample pad which then flow through the conjugate pad. The labelled antigen-antibody complex bind the nitrocellulose membrane and from visible line at the test line. The intensity of the test line determine the concentration of antigen present in the sample (Galatas *et al.*, 2020). For more descriptions about vertical and lateral flow (Fig. 3 & 4).

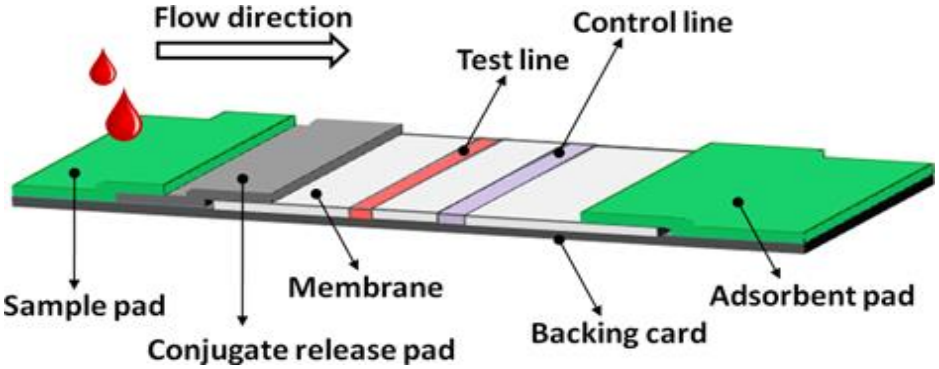

Figure 2: Lateral flow assay for antigen detection (Koczula & Gallotta, 2016)

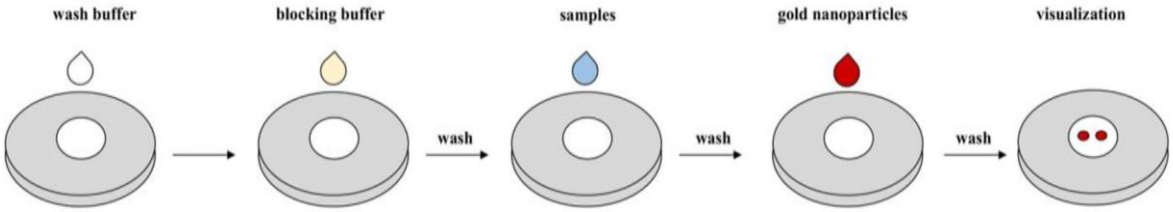

Figure 3: Vertical flow assay for antigen detection (Lei *et al.*, 2022)

Moreover, dual path platform technology was introduced and involves the use of strip and buffer solution. The strip contains two lines, the control and test line. Buffer used to facilitate detection of malaria parasite. The platform is able to capture antibodies or antigens in the blood of patient. And it is easier to use and require minimum training and provide results within 15-20 minutes (Cunningham *et al.*, 2019). It is improving the accuracy of the test using the control and test lines to ensure that the test is working properly. The platform assay has the benefit of detecting multiple strains of malaria at once allowing accurate diagnosis and treatment plan. Additionally, detecting multiple strains can help track the spread of different strains of malaria at different regions, which can aid in development targeted prevention and control strategies.

Despite the advantages of RDTs, the technique offers some disadvantages especially in low malaria transmission where sensitivity decreases and the chance of detecting the true positive also declines. In order to overcome this, it is important to use malaria RDTs which are more sensitive in low, mid, and high malaria parasite transmission (Boyce & O'Meara, 2017). Traditionally RDTs are already sensitive enough to detect malaria antigen in the blood sample. However the high sensitive RDT have ability to detect even low levels of malaria parasites in the blood and increase

accuracy of malaria diagnosis in cases where the parasite load is very low. While ultrasensitive RDTs are more sensitive since they have ability to detect small number of parasite and produce a lower threshold. Moreover, malaria parasite has an ability to mutate leading to deletion of gene encoding the histidine rich protein (HRP) which is marker for the presence of malaria parasite in the patient's blood.

2.4 Histidine Rich Protein 2 Gene Deletion and Detection Limit

Gene deletion refers to the loss of one or more genes from the genome from an individual or population which can occur naturally or artificially means such as genetic engineering. These genes are responsible for particular proteins which perform specific functions. Malaria rapid diagnostic test (mRDT) as diagnostic tools which detects malaria parasites (Fontecha *et al.,* 2018), histidine rich protein as the marker for the presence of malaria parasite in the blood of the patient. mRDT is designed to detect histidine rich protein (HRP2) produced by *Plasmodium falciparum* and histidine rich protein3 (HRP3) which produces by non-falciparum species (Schindler *et al.,* 2019).

During infection of the red blood cells, a larger amount of histidine rich protein is produced and detected by mRDTs (Prosser *et al.,* 2021). However, malaria parasite have the ability to mutate leading to deletion of genes encoding the HRP during infection making the parasite undetectable by rapid diagnostic tests that rely on detecting these proteins (Vera-Arias *et al.,* 2022).

The mRDTs are immunochromatography and the most essential tools to the most health facilities which assist in malaria case management and diagnosis. But during infection the parasite undergoes mutation leading to deletion of the gene which is encoding HRP. This makes the test fail to detect the particular gene hence encountering false negative results (Das *et al.,* 2018). Also gene deletion weakens the performance effectiveness of the kit (Kaaya *et al.,* 2022).

Before stating that the performance of mRDT is negative we need to confirm if there is HRP2/3 gene deletion in order to avoid giving patients incorrect diagnosis and treatment (Pati *et al.,* 2018). Additionally, knowing the prevalence at the study area it can provide the information into the pathogenicity of the parasite at a particular area. It also helps to track the spread and evolution of malaria parasite strains (Oreh *et al.,* 2022). Prevalence may differ due to methodological approaches.

The working accuracy of a malaria rapid diagnostic test depends on how sensitive it is to detect parasitemia to asymptomatic patients. Most malaria RDTs detection limit is approximately 100–200 parasites/μL (Mwesigwa *et al.,* 2019). Generally a pool of several studies reported diagnostic

performance of mRDTs with sensitivity of 42% (95%CI: 25–62%) and 61% (95%CI: 47–73%) for conversional-RDT (co-RDT) and ultra-sensitive-RDT (us-RDT) respectively, with specificity of 99% (95%CI: 98–100%) for co-RDT, and 99% (95%CI: 96–99%) for us-RDT (Yimam *et al.*, 2022).

Hence effort has been put to improve the diagnosis by evaluating performance of novel malaria RDTs to improve the control and elimination strategies (Wu *et al.*, 2015). To supplement the effort, the current study is also aiming to determine the new RDTs detection performance when compared to those which have improved and are in the market.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Design

This study was a cross-sectional study nested in the big project known as clinical, analytical and blood Type Studies. Clinical arm aimed at evaluating the clinical performance of the One Step Malaria *(P. f/ P. v*) Tri-line and One Step Malaria (*P. f*) rapid diagnostic tests and generating evidence based technical specification data to support application for WHO pre-qualification of IVDs. The evaluation aims at determining the sensitivity of the test that, is the ability of the test to identify individuals who have diseases or conditions that the test is designed to detect. Clinical performance also based on specificity as ability of the test to identify individuals who do not have the disease.

In the analytical study arm, we examined whether there are performance differences amongst the One Step *P.f/P.v* mRDTs Tri-line and One Step Malaria *(P. f)* tested in blood collected from juvenile patients aged 12-17 years old who also were co infected by other diseases. Samples with co-infection were selected based on a national algorithm for diagnosis of diseases in health care facilities. The blood type arm aimed to examine whether there is significant different between blood types (anticoagulant) used when whole blood collected directly from venous and preserved in different anticoagulants (EDTA, Heparin, Sodium Citrate) and tested using One Step Malaria *P.f/P.v* Tri-line One Step Malaria *(P. f)* rapid diagnostic tests.

For malaria detection, finger prick and venous blood was obtained from participants presented to the health care facilities in the selected study sites with fever or a history of fever within 48 hours or an illness that the attending doctor suspected might be due to malaria infection. The study participants were children above five years and adults The study was conducted from July to October 2020 where patients were enrolled and samples collected, processed, analysed, and data generated for statistical analysis.

3.2 Study Area

The study was carried out at Bagamoyo and Kibiti districts. From Kibiti district, samples were collected from (Bungu and Mtawanya health facilities) which represents a stable and high malaria transmission and Bagamoyo district (Fukayosi, Yombo, Kongo and Mkenge health facilities) with low to moderate malaria transmission. Kibiti district is located in the coast region of Southeast Tanzania. It lies at southern of the Dar es Salaam and found between 7.7218 $^{\circ}$S and 38.9375°E

along the Dar es Salaam-Lindi and Mtwara highway. The district is part of a hot, humid, coastal plain with varying tropical climatic conditions (Ngasala *et al.,* 2019; Runge *et al.,* 2020). The study area is characterized by seasonal rainy and vast water bodies which facilitate transmission of malaria. The area normally gets rain twice a year; the short rainy season occurs during September and October, and the longest rainfall happens from February to May. Malaria transmission is still a challenge in this area especially during and after the period of long rain season. About 96% of all malaria cases are caused by *Plasmodium falciparum,* while 4% are due to *P. malariae* and *P. ovale*.

Bagamoyo District the rainy season starts from March to May, with a second season from November to December, although occasional rain occurs at all times of the year. Average rainfall is 1200 to 2100 mm per year. The study area covers grassland vegetation and subsistence agriculture throughout the year. Average temperature for the region is about 28°C (Salim *et al.,* 2015). The main activities are agriculture and are supported by the Ruvu River and its tributaries. The study area has moderate perennial malaria transmission with higher transmission intensity towards the west of the study area. Malaria disease is almost entirely due to *Plasmodium falciparum,* with *P. malariae* and *P. ovale* occurring in less than 5% of infections with *P. falciparum* (Mwangoka *et al.,* 2009).

Based on regional censor which were conducted in 2022, the report shows that population at Pwani was 2 024 947 (URT, 2022) with 6.7% prevalence of malaria in children (TDHS, 2022).

3.3 Study Population

A total of 1630 participants (1175 *P.f* negative and 455 *P.f* positive) above five years children and patients with fever within 48 hours who meet enrolment criteria at health facilities were randomly selected and tested for malaria using both the registered SD BIOLINE and mRDT tests kits, InTec PRODUCT, INC Company, China. Sample size determination was based according to TSS-3 Technical Specifications Series for submission to WHO Prequalification-Diagnostic Assessment: Malaria rapid diagnostic tests guideline where, a minimum of 1500 have to been rolled (WHO, 2017). The study was implemented by three arms named: (a) clinical (1104 negative and 410 positive), (b) analytical (21 negative, 20 negative with other diseases and 20 positive), and (c) blood type (30 negative and 25 positive, each patient parallel tested with finger prick, venous whole blood in EDTA, Heparin and Sodium citrate tubes).

3.4 Evaluation Procedures

The three malaria diagnostic techniques: The mRDTs, microscope, and real-time PCR were used to diagnose malaria blood samples.

3.4.1 Evaluation of Test Kits

Blood from both finger prick and venous were used for mRDT test kits (One Step Malaria (*P.f/P.v*) triple line and One Step Malaria (*P.f*)) evaluation. According to the manufacture test procedure, test cassettes were placed on a clean and level surface then labelled with specimen identification number. The 5 µl of venepuncture whole blood transferred to the specimen well (S) of the test cassette, then 3 drops of buffer added to a buffer well (B) immediately. Results were read in 20 minutes.

Results were stated *p.f* positive if one line appeared in a control region and one line appeared in the *p.f* region or pan line for *P.f/P.v*. Negative results stated if one coloured line appeared in the control region. The results stated invalid if the control line fails to appear in a control (C) region.

In addition, venous whole blood collected in the EDTA tubes were used for microscopy slide preparation using the laboratory SOP and confirmed by qPCR. For patients tested negative for malaria, other available diagnostic tests were performed to establish the cause of fever. Trained lay providers (TLP), "trained health care worker (THCW) and technicians participated as operators for mRDT test kits evaluation according to manufacturer's instructions two independent readers examined and gave out results for each tested cassette blindly.

3.4.2 Malaria Parasite Detection by Microscope

Before detection of malaria parasites by microscope, thick and thin smears were prepared according to the Ifakara Health Institute laboratory standard operating procedure (SOP). Thick smear prepared by pipetting 6 µL of blood sample collected in EDTA tube onto the left side of the glass slide. A corner of another slide used to spread the drop in a circular pattern. Then the slides layer flat and allowed the smears to dry thoroughly for 30 minutes at room temperature. Note thick smear was not fixed.

Thin smear were prepared by pipetting 2 µL of blood samples collected in an EDTA tube and dropped on top of the pre-cleaned, labelled slide, near its frosted end. Another slide was brought at a 30-45° angle up to the drop, so as to allow the drop to spread along the contact line of the 2 slides. Quickly the upper slide was pushed toward the unfrosted end of the lower slide. The smear was left to dry. Then the thin smear was fixed by dipping them in 1ml of absolute methanol. The

thin blood film was covered with methanol with a clean glass slide so that that methanol does not spread and come into contact with the thick blood film. The remaining methanol was removed from the edge of the slide by wiping using tissue paper then the slide placed on a draining rack with the frosted side on top and allowed to dry for 2 minute.

Blood smears were stained by 10% Giemsa stain for screening of asexual parasites and gametocytes. Slides were declared negative if no parasites observed in 100 high power microscopic fields. Asexual parasites and gametocytes were counted against 200 and 500 white blood cells, respectively and the final parasite count was used to calculate the parasite density and documented. Parasite density was calculated according to the laboratory standard procedures, as per WHO standard. Quality control for positive and negative slides was done on a daily basis. Samples tested positive by microscope and verified by PCR, were selected for clinical sensitivity study. Information on other causes of fever was collected from the patients tested negative for plasmodium and the patient was selected for clinical specificity study.

3.5 Molecular Techniques

3.5.1 DNA Extraction

Genomic DNA was extracted from the whole blood using Quick-gDNA Blood MiniPrep Kit from Zymo Research (Inqaba Biotec, ZR D3025), following the manufacturer's instructions. To prepare 1 mL of 5x PlasQ Primer Probe Mix following volumes as illustrated in Table 1 and was pipetted into a 1.5 mL Eppendorf tube (Schindler *et al.*, 2019).

Table 1: Volume for qPCR Primer Probe Mix

Primer	Stock concentration [µM]	Final concentration in 5x Primer Mix	Volume in µL	References
Pspp18S F	100	2	20	Schindler *et al*. (2019) Kamau *et al.* (2013)
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	

3.5.2 Master Mix Preparation

The master mix was prepared for qPCR assay for quantification of *Plasmodium falciparum* according to the number of samples to be analyzed per day together with an additional of 10% error. The volume was prepared as indicated in Table 2.

Table 2: Volume for Master Mix preparation

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

3.5.3 Polymerase Chain Reaction Procedure for Sample Amplification

All qPCR Master Mix components was pipetted into a 1.5 mL Eppendorf tube. The master mix was gently mixed by vortexing. The 8 µL of the qPCR Master Mix was transferred to each well. The 2 µL Molecular biology grade H_2O added to the NTC samples. The qPCR plate transferred to the DNA addition bench. The 2 µL template DNA added to each well. The 2 µL of PC added in duplicates. The 2 µL of NC added in duplicates too. The final reaction volume including the DNA template was 10 µL.

The qPCR assay consists of two independent Plasmodium targets that were combined in a multiplex assay. The Pan-Plasmodium 18S rRNA sequence (Pspp18S) and the *Plasmodium falciparum* specific var gene acidic terminal sequence (P.fvarATS) were targeted. The human RNaseP sequence was targeted as an internal control to assess the quality of DNA extraction and qPCR amplification. The use of two Plasmodium targets ensures high specificity and increase the sensitivity of *Plasmodium falciparum* and non-falciparum species detection.

3.5.4 The qPCR Assay for Quantification of *Plasmodium falciparum*

Real-time qPCR assays were performed using the Bio-Rad CFX96 Detection System and pre-analysis by using CFX Manager Software (Bio-Rad, version 3.1). The thermal profile used for qPCR was as follows: 1min at 95°C; 45 cycles of 15 s at 95°C; 45 s 57°C.

3.5.5 Relative Standard Curves

The *P. falciparum* genomic DNA (WHO reference from NIBSC) was used to generate the relative standard curves for comparison of qPCR and test kits limit of detection. Genomic DNA from the

100 000 parasite/µl sample was serially diluted into a different range of concentrations up to the lowest one concentration of 0.001p/ µL, as shown in Table 3.

Table 3: Concentrations for relative standard curves

DNA sample concentration (NIBSC…100000parasite/µl)	Volume of sample required(µl) from previous dilution	Volume of water required(µl)	Final volume(µl)	Parasite density(P.f/ µl)
S01	Start	Start	6	100 000
S02	Start	Start	200	10 000
S03	100	100	200	5000
S04	80	120	200	2000
S05	100	100	200	1000
S06	100	100	200	500
S07	80	120	200	200
S08	100	100	200	100
S09	100	100	200	50
S10	80	120	200	20
S11	100	100	200	10
S12	100	100	200	5
S13	80	120	200	2
S14	100	100	200	1
S15	100	100	200	0.5
S16	80	120	200	0.2
S17	100	100	200	0.1
S18	20	180	200	0.01
S19	20	180	200	0.001

Key: SO1-S19, were dilution number. Start: Initial concentration before dilution

3.5.6 qPCR for Detection of HRP-2 Gene Deletion

The HRP-2 gene deletion was identified from samples that were mRDTs negative but positive by microscopy and qPCR. To evaluate the effectiveness of DNA extraction and qPCR amplification, the internal control gene for P.frnr2 (P.F3D7 1015800) with sequences of

AGTATCCAAAACACTATAATTCCAAGTAC (Forward)

and ATTTTCTCCTTTCTTAACAGTTTCTTCC (Reverse)

respectively, were used. Also gene deletion marker, P.frp2 (P.F3D7 0831800) with sequences of GTATTATCCGCTGCCGTTTTTGCC forward and TCTACATGTGCTTGAGTTTCG reverse were chosen respectively. Utilizing the Bio-Rad CFX96 detection system and the CFX Manager Software, real-time qPCR assays were performed. Master Mix was prepared and following the thermal profile was used for qPCR: The 5 minutes at 95°C, 45 cycles, 15 seconds at 95°C, and 35 seconds at 57.5°C. Primers probe mix was prepared as shown in the Table 4.

Table 4: Primer probe mix volume for hrp2 gene deletion

Primers and probe	Stock concentration [µM]	Final concentration in 5x Primer Mix	Volume in µL
IC-P.fRNR2E2 F	100µM	1	10
IC-P.fRNR2E2 R	100µM	1	10
IC-P.fRNR2E2 CY5	100µM	0.5	5
P.fHRP2 F	100µM	2	20
P.fHRP2 R	100µM	2	20
P.fHRP2 TxRd	100µM	1	10
Molecular biology grade H_2O			675

Table 5: Master Mix preparation for hrp2 gene deletion detection

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

3.5.7 Polymerase Chain Reaction Procedure for Sample Amplification

All qPCR Master Mix components was pipetted into a 1.5 mL Eppendorf tube. The master mix was gently mixed by vortexing. The 8 µL of the qPCR Master Mix was transferred to each well. The 2µL Molecular biology grade H_2O added to the NTC samples. The qPCR plate transferred to the DNA addition bench. The 2 µL template DNA added to each well. The 2 µL of PC added in duplicates. The 2 µL of NC added in duplicates too. The final reaction volume including the DNA template was 10 µL. The qPCR assay consists of two independent Plasmodium targets that were combined in an assay. The *P.f*rnr2e2 (P.F3D7_1015800) which was targeted as an internal control to assess the quality of DNA extraction and qPCR amplification and *P.f*rp2 (P.F3D7_0831800) as the *Plasmodium falciparum* gene deletion detection.

The qPCR assays were performed using the Bio-Rad CFX96 Detection System and pre-analysis by using CFX Manager Software (Bio-Rad, version 3.1). The thermal profile was used for qPCR as follows: The 5 min 95°C followed by 45 cycles then 15 s at 95°C and 35 s at 57.5°C.

3.6 Detection Performance

The detection performance of the tested RDTs were determined with reference to microscope and qPCR results by combining data from two assays. Firstly, P. falciparum genomic DNA (WHO

reference from NIBSC, with 100 000 parasite/µl) was serially diluted into a different range of concentrations up to the lowest concentration of 0.001 p/ µL to generate the relative standard curves of which were used to obtain the slope and y-intercept for actual estimation of parasite density as shown in Table 3. The parasite density established from standard curve were finally used for comparison of qPCR and test kits performance detection.

Secondly, data were established from microscope method and blood slides were examine by expert technicians who work at the IHI-Bagamoyo laboratory, which is ISO.15189:2022 accredited by SADCAS and the microscope method was regarded as the gold standard. Only the samples with qPCR positive and blood slides positive results were considered "true positive" and used for generation of parasites range that were used for RDT detection performance. Six categories:

(i) <=10 p/µL,

(ii) 11-50 p/µL,

(iii) 51-100 p/µL,

(iv) 101-200 p/µL,

(v) 201-2000 p/µL, and

(vi) & >= 2000 p/µL, were used to group the detection performance

3.7 Data Management

Thick and thin blood smears as well as qPCR was conducted at reference laboratory of IHI in Bagamoyo for the purpose of quality control. Laboratory scientists and technicians working at reference laboratory were blinded to the tested *P.f/P.v* and *P.f* mRDT and registered mRDTs kits results. Results of blood slides and *P.f/P.v* and *P.f* mRDT were also blinded to laboratory scientist who performed qPCR. Microscope slides were examined by competency microscopists. *P.f/P.v* and *P.f* mRDT under PQ were performed according to manufacturer's instructions InTec Product, Inc Company, China. For assessing inter-observer variability, *P.f/P.v* and *P.f* mRDT results were prepared and independently interpreted by trained lay provider (TLP) or trained health care workers (THCW) or and laboratory professional (Technicians) as described in the protocol. Patients were treated in accordance with national guidelines if tested positive by smear microscopy or by registered mRDTs SD BIOLINE (lot # 05EDE011A) manufactured by SD Standard Diagnostic, Inc., Alere company.

3.8 Statistical Analysis

All results generated during the evaluation were entered into an excel sheet. The final datasets contained 35 variables, but for the purposes of this dissertation only demographic characteristics and testing results were included in the analysis. The microscope, qPCR, and mRDT (*P.f*, and *P.f/P.v*) test results were analysed. It was possible to access the consistency of positive and negative results for all tests, as well as those that did not show similarity. When the qPCR and microscope results were similar in terms of positivity and negativity, sensitivity and specificity were established. The STATA version 15, Texas college software was used to determine sensitivity, specificity as well as detection performance of the One Step Malaria *(P.f/P.v)* triple line and One Step Malaria (*P.f)* using microscopy and qPCR as the reference. The 2 by 2 table was used to calculate the sensitivity and specificity. The sensitivity, specificity, negative predictive value (NPV), and positive predictive value (PPV) were calculated by comparing mRDT (*P.f*, and *P.f/P.v)* results to the microscopy and qPCR as reference methods. The output includes sensitivity, specificity, NPV, and PPV with associated 95% confidence intervals where by the reference standard is qPCR result from the same patient.

3.9 Ethical considerations

The study was approved by the Institution Review Board (IRB) and National Ethical Committee for Health Research, in Tanzania. Study protocols used complied with International Conference on Harmonization Good Clinical Practice (ICH-GCP) guidelines, good laboratory clinical practice (GCLP) and standard operation procedures (SOPs). All personnel worked in this study both in the laboratory and field clinics were trained on the study protocol and SOPs. All study participants were consented and signed a written informed consent before screened and enrolled into the study. Parents or guardian provided consented for their children and adolescence were asked to assert as well. For the illiterate individuals, a witness selected by the participants signed the consent on behalf and the participant provided a fingerprint.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Results

A total of 1630 (1175 *P.f* negative and 455 *P.f* positive) patients seeking medical care and met the enrolment criteria at health care facilities, were tested for malaria using both the registered mRDT (SD BIOLINE), the One Step Malaria (*P.f/P.v*) Tri-line One Step Malaria (*P.f),* microscope and qPCR as reference. The mRDT SD BIOLINE results were used to manage patients at the health facilities because the One Step Malaria (*P.f/P.v*) Tri-line One Step Malaria (*P.f)* results were for research purposes only.

The study population comprised 62.1% (1012/1630) female participants and 34.5% (562/1630) male participants. The 10.98 % (179/1630) and 23.5% (383/1630) of male participants were malaria positive and negative respectively and 15.5 % (253/1630) and 46.6 % (759/1630) of female participants were malaria positive and negative respectively. Information on gender for 56/1630 (3.4%) was missing. The study participants were categories in different age group of children , young adults, adults and old individuals. The characteristics of the study demographic are summarized in Table 6.

Table 6: A summary of the Study Demographic Characteristics

Age category	Gender	Positive	Positive %	Negative	Negative %	Subtotal number	Malaria Prevalence%
5-18 years	Male	127	59.3	87	40.7	214	32.2
	Female	146	46.9	165	53.1	311	
19-45 years	Male	45	15.9	238	84.1	283	54
	Female	97	16.2	501	83.8	598	
46-65 years	Male	7	11.7	53	88.3	60	9
	Female	6	7	80	93	86	
>65 years	Male	0	0	5	100	5	1.3
	Female	4	23.5	13	76.5	17	
Unspecified:	NA	23	41.1	33	58.9	56	3.4
Grand total		455		1175		1630	99.9

4.1.1 Sensitivity and Specificity One Step Malaria RDT Tests

Sensitivity, Specificity, positive predictive value (PPV) and negative predictive value (NPV) of

One Step Malaria *P.f* mRDT and One Step Malaria *(P.f/P.v*) Tri-line test mRDTs were obtained as indicated in Table 7. To determine the performance of One Step Malaria *P.f/P.v* Tri-line mRDTs and One Step Malaria *P.f* mRDTs, sensitivity and specificity of the kits were calculated with reference to blood smear results that were confirmed by qPCR. All test results were analyzed to calculate the clinical sensitivity and specificity of the test kits. The sensitivity and specificity values were calculated from true and false positives outcomes of the test kits against the standard method which is microscope and qPCR using the 2 by 2 Table.

Table 7: The 2 by 2 table demonstrates how the sensitivity and specificity of the test kits were calculated

	Microscopy		
One step malaria	Positive	Negative	Total
Positive	a	b	a+b
Negative	c	d	c+d
Total	a+c	b+d	

Key: Sensitivity=a/ a+c and Specificity=d/b+d; PPV=a/ a+b and NPV=d/ c+d

Table 8: Showing data for Sensitivity, Specificity, PPV and NPV of One Step Malaria P.f mRDTs

One Step Malaria *P.f* Test				
One Step Malaria *P.f* Test	**P.f: Microscopy (B/s) and qPCR as composite reference method**			
	Finger prick		**Venous**	
	***P.f* Positive**	***P.f* Negative**	***P.f* Positive**	***P.f* Negative**
P.f Positive	359	10	358	11
P.f Negative	17	523	18	521
Total	376	533	376	532
Invalids		0		1

Table 9: Showing data for Sensitivity, Specificity, PPV and NPV of One Step Malaria P.f/P.v Tri-line mRDTs

One Step Malaria *P.f* Test	**One step *P.f* /*P.v*: Microscopy (B/s) as reference method**			
	Finger prick		**Venous**	
	***P.f* Positive**	***P.f* Negative**	***P.f* Positive**	***P.f* Negative**
P.f Positive	367	60	367	56
P.f Negative	16	1036	15	1037
Total	383	1096	382	1093
Invalids		**1**	**1**	**2**

Table: 10 Table 8c: Showing data for Sensitivity, Specificity, PPV and NPV of SD Bioline mRDTs

SD Bioline	Reader	
	***P.f* Positive**	***P.f* Negative**
P.f Positive	357	12
P.f Negative	19	521
Total	376	533
Invalids		0

The results showed that, there was no different in sensitivity and specificity of One Step Malaria *P.f/P.v* Tri-line mRDTs when finger prick blood were used [95.7 % (93.2% to 97.6% CI) and 97.9% (96.3% to 99.0% CI)], respectively and when venous blood were used sensitivity and specificity were 96.0% (93.5% to 97.7% CI) and 98.3% (96.8% to 99.2% CI) respectively. Similar finding was observed for the One Step Malaria *P.f* test mRDTs [sensitivity, 95.5 % (92.9% to 97.3%) and specificity, 98.1% (96.6% to 99.1% CI)] when finger prick blood were was used and [sensitivity, 95.2% (92.5% to 97.1% CI) and specificity, of 97.9% (96.3% to 99.0% CI)] when venous blood were used. Additionally, the sensitivity and specificity of SD Bioline mRDTs that are currently in the market and used in the health facilities was [94.95% (92.22% to 96.93% CI) and 97.75% (96.10% to 98.83%)] (Table 11, 12 and 13).

Table 11: Sensitivity and Specificity of One Step Malaria P.f/P.v Tri-line Test mRDT in reference to blood smears and qPCR when all the discrepancies were removed

Parameter	**Fingerpick**	**95% CI**
Sensitivity	95.82%	93.30% to 79.59%
Specificity	94.53%	93.01% to 95.80%
PPV Positive Predictive Value	85.95%	82.69% to 88.67%
Negative Predictive Value	98.48%	97.57% to 99.05%
Parameter	**Venous**	**95% CI**
Sensitivity	96.07%	93.61% to 97.79%
Specificity	94.88%	93.40% to 96.11%
PPV Positive Predictive Value	86.76%	83.54% to 89.43%
Negative Predictive Value	98.57%	97.68% to 99.13%

Table 12: Sensitivity and Specificity of One Step Malaria P.f Test mRDTs in reference to blood smears and qPCR when all the discrepancies were removed

Parameter	Fingerpick	95% CI
Sensitivity	95.5%	(92.9% to 97.3%)
Specificity	98.1%	(96.6% to 99.1%)
PPV Positive Predictive Value	97.3%	(95.1% to 98.5%)
Negative Predictive Value	96.9%	(95.1% to 98.0%)
Parameter	**Venous**	**95% CI**
Sensitivity	95.2%	(92.5% to 97.1%)
Specificity	97.9%	(96.3% to 99.0%)
PPV Positive Predictive Value	97.0%	(94.8% to 98.3%)
Negative Predictive Value	96.7%	(94.9% to 97.9%)

Table 13: Sensitivity and Specificity of SD Bioline mRDT in reference to (B/s) and qPCR as composite reference method

Parameter	Value	95% CI
Sensitivity	94.95%	92.22% to 96.93%
Specificity	97.75%	96.10% to 98.83%
Positive Predictive Value	96.75%	94.44% to 98.12%
Negative Predictive Value	96.48%	94.65% to 97.70%

4.1.2 Detection Performance of mRDT based on Blood Smears Results Confirmed by qPCR

Detection performance of mRDT based on Blood Smears results confirmed by qPCR One Step Malaria P.f/P.v Tri-line, One Step Malaria P.f rapid test kits and SD Bioline mRDTs performance were similar across the parasite density ranges. At the parasite density range of above 100 p/µl, the detection agreement of P.f, and P.f/P.v mRDTs as well as the SD Bioline mRDTs were over 75%. However, at the parasite density range of between 200 and 2000 p/µl, the detection agreement of P.f, and P.f/P.v mRDTs as well as the SD Bioline mRDTs were over 95%. The percentage agreement for sample with very higher parasites density (> 2000 p/µL) were above 99%. (Table 14 and 15).

Table 14: Detection performance of One Step Malaria P.f/P.v and P.f mRDTs in specified range of parasite densities categorized by to Blood Smear results and confirmed by qPCR methods

Parasite density	*P.f/P.v* mRDT				*P.f* mRDT			
	Positive number	Reference	Agreement%	Discrepancy	Positive number	Reference	Agreement%	Discrepancy
<10 p/ul	0	0	100	0	0	0	100	0
11p to 50 p/ul	3	4	75	1	2	4	50	2
51 to 100 p/ul	6	12	50	6	6	12	50	6
101 to 200 p/ul	13	17	76.5	4	13	17	76.5	4
200 to 2000 p/ul	65	69	94.2	4	65	69	94.2	4
>2000 p/ul	273	274	99.6	1	272	274	99.3	2
Grand total	360	376		16	358	376		18

Table 15: Detection performance of the SD Bioline RDTs in specified range of parasite densities categorized by Blood Smear and confirmed by qPCR

Table 15: Detection performance of the SD Bioline RDTs in specified range of parasite densities categorized by Blood Smear and confirmed by qPCR

Parasite density	Positive number	Reference	% of agreement	Discrepancy
<10 p/µl	0	0	100	0
11to 50 p/µl	2	4	50	2
51 to 100 p/µl	6	12	50	6
101 to 1000 p/µl	13	17	76.5	4
1001 to 2000 p/µl	65	69	94.2	4
>2000 p/µl	271	274	98.9	3
Grand total	357	376		19

4.1.3 Histidine Rich Protein2 (HRP-2) gene detection

The 376 samples were malaria positive by both blood slides and qPCR. 44 out of 376 samples that were positive by both qPCR and microscopy and, negative by One Step *P.f/P.v* Tri-line, One Step Malaria *P.f* only and SD Bioline mRDTs and were further analyzed for HRP-2 gene deletion by qPCR assays. The 7/44 (15.9%) samples gave positive results for HRP-2 gene, implying that the gene was there. However, 37/44 (84.1%) were negative for the HRP-2-gene. The general proportion of HRP-2 gene deletion reported from the current study was 9.8 % [7.84 -11.76] (Table 11). The mean parasite densities for these samples in blood slide and qPCR were 1975.1 p/µl and 3219.9 p/µl, respectively.

Table 16: Histidine Rich Protein 2 gene detection for both One Step Malaria P.f and P.f/P.v test kits which presented negative results with reference to blood smear and confirmed qPCR

Number of participants	**qPCR+/ BS+/**	**qPCR+/BS+/RDT-**	**qPCR+/ BS+/ RDT-/ , No HRP-2 gene deletion**	**qPCR+/ BS+/ RDT-/ with HRP-2 gene deletion**	**Proportion of HRP-2 gene deletion (95% CI)**
	376	44	7	37	37/376 (9.8 %) [7.84 to 11.76]
Mean parasite density for blood slide = 1975.1			Mean parasite density for qPCR = 3219.9		

4.1.4 Analytical Study Results

In analytical study arm, we aimed to examine whether there is a performance difference amongst the One Step *P.f/P.v* mRDTs Tri-line and the One Step Malaria *P.f* tested in blood collected from juvenile patients aged 12-17 years old. Table 12 indicated 28 number of malaria positive sample detected by both BS/qPCR and mRDTs that was also positive by other infections as shown in Table 17

Table 17 Performance of the One Step P.f/P.v mRDTs Tri-line and one step P.f mRDTs in relation to diseases other than malaria and malaria coinfections

	BS/qPCR as Reference		
Diseases	**Malaria Positive**	**Malaria Negative**	**Total**
URTI	8	6	14
UTI	8	0	8
TONSILITIS	0	1	1
DENTAL CARIES	1	0	1
DIARRHOEA	2	1	3
enteric FEVER	1	1	2
PNEUMONIA	3	0	3
HIV	3	1	4
TB	2	0	2

However, a number of patient presented in the health facilities for analytical study had malaria test negative results. We opted to investigate other causes of fever for our documentations. Table 12 above, represent other cause of illnesses that led patients to seeking medical care, however malaria infection was observed to be the main cause of fever in the communities, followed by upper respiratory truck infection and urinary truck infection. Few patients had co infection of malaria and HIV, and malaria and Urinary truck infection and co-morbidities didn't affect the performance of the test kits. From the Table 12 it is observed that, there is no interference in the performance accuracy of the One Step Malaria *P.f/P.v* Tri-line mRDT and One Step Malaria *P.f* mRDTs by the common diseases that are in detected in the malaria endemic areas.

4.1.5 Blood Type (Anticoagulant) Study Results

We investigated results generated by our two independent readers when blood collected in different anticoagulants were tested by the mRDTs. The data suggests that there was a highly significant correlation between the readers and the blood types, the anticoagulant. There was no significant different between blood types used when whole blood collected directly from the finger prick or venous and preserved in different anticoagulants and tested using One Step *P.f/P.v* mRDTs Tri-line and the One Step Malaria *P.f.* Additionally, results generated from different intended user were similar in all blood type, that means different anticoagulant doesn't affected the performance of the test kit, results are as shown in Fig. 2a and 2b

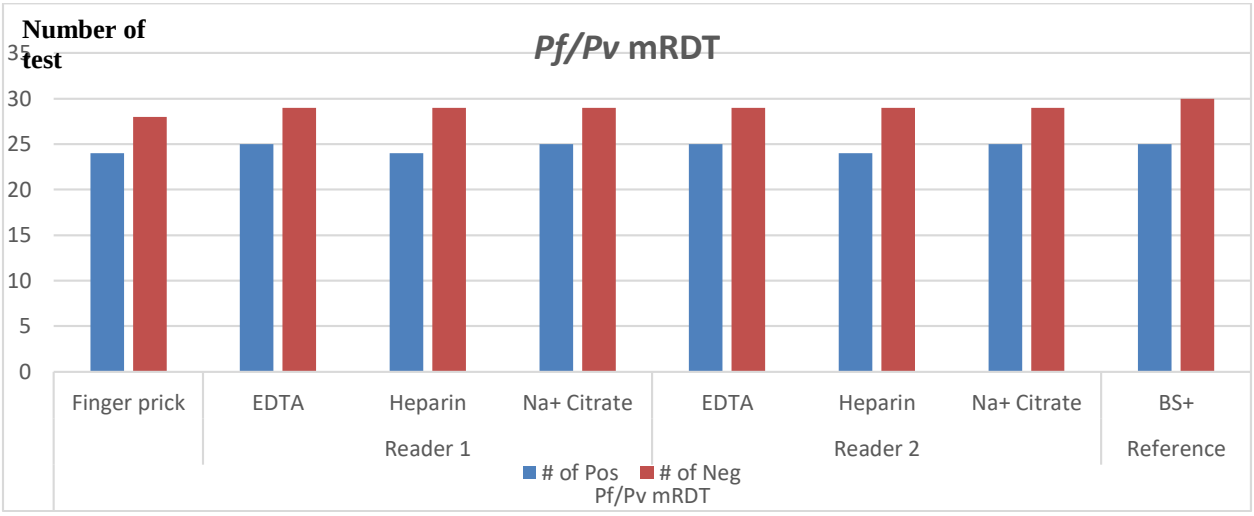

Figure 4: Histograms representing number of positive and negative results for P.f/P.v mRTDs obtained when different anticoagulant (blood type) were used for testing by different readers when blood smear and qPCR was used as reference

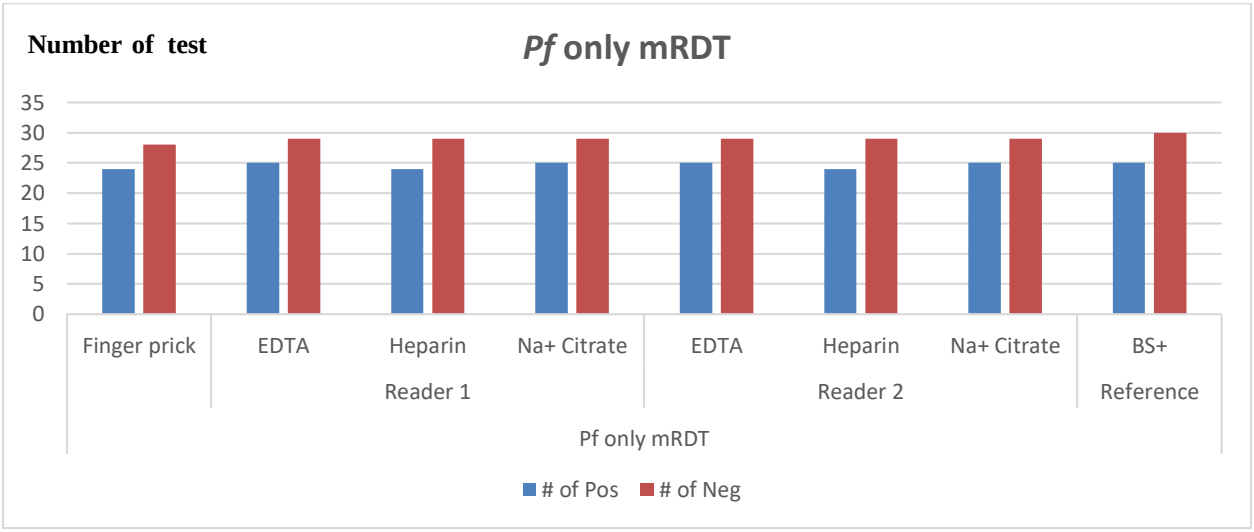

Figure 5: Histograms representing number of positive and negative results for P.f mRDTs obtained when different anticoagulant (blood type) were used for testing when blood smear and qPCR were used as reference

4.2 Discussion

The current study demonstrated that the sensitivity and specificity of the new One Step Malaria *P.f/P.v* Tri-line and *P.f* mRDTs was similar to that of the standard mRDT kit (SD Bioline) in the field evaluation demonstrating its potential in contribution in fight against malaria in endemic areas. Sensitivity and specificity of other novel mRDTs have also been compared to the standard mRDT kits (SD Bioline) which have been on the market for decades (Willie *et al.,* 2018). The performance of the evaluated test kits in this study are in agreement with the results obtained from other mRDTs that are currently in the market (Slater *et al.,* 2015). The scored sensitivity and specificity were similar to sensitivity and specificity reported by other mRDT kits available in the market (Charpentier *et al.,* 2020; Eticha *et al.,* 2020; O *et al.*, 2021).

The malaria RDTsand microscope are the most common tests that are routinely used for the management of malaria cases in Tanzania, despite the fact that, thin and thick blood slides remained as gold standard for malaria diagnosis to date (Mathison & Pritt, 2017; Belachew *et al.*, 2022). Malaria diagnostic tools with sufficient limit of detection to identify even the asymptomatic and reservoir with low parasitemia but enough to transmit is vital in fight against malaria and will definitely supplement in the existing malaria control strategies (Mbabazi *et al.,* 2015). Despite the fact that qPCR is more sensitive, this technique has limited usefulness in the standard care, hence is mainly used for confirmation of malaria parasite species (Global Malaria Programme, 2017).

Interestingly, One Step Malaria *P.f/P.v* Tri-line and *P.f* mRDTs detected up to 50% of samples with parasite density as lower as 11parasites/µl and over 75% of samples in parasite ranges of 101-200 parasites/µl similar to standard mRDT kit (SD Bioline). The results from this study, showed that, both One Step Malaria *P.f/P.v* Tri-line and One step *P.f* test kits have capability of detecting proportion of cases with very low parasite density (~ 11parasites/ µl). The results are in agreement with the most mRDT that are currently in the market which have reported to have capacity to detect correctly parasite density of below 100 parasite/µl (Mwesigwa *et al.,* 2019; Wu *et al.,* 2015; Taylor *et al.,* 2019).

The sensitivity of mRDTs depend solely on amount of antigen produced by individuals during course of infection (Ogunfowokan *et al.,* 2020). The amount of HRP2 may vary in the blood circulations depending on many factors amongst which includes, P.fhrp2/3 gene deletions. Histidine Rich Protein-2 (HRP-2) is produced in abundance by *Plasmodium falciparum* and it is highly stable (Kong *et al.,* 2021). In this study, we determined the HRP-2 gene deletion in samples that were tested negative by mRDTs but positive by microscope and qPCR. Approximately ten percent (9.8%) of all *P. falciparum* positive samples had HRP-2 gene deleted which causes false negative in some participants. According to WHO if estimated HRP-2/3 gene deletion is higher than 5%, then there is a high confidence that the proportion of false negative mRDT results in symptomatic *P. falciparum* patients is caused by P.fHRP-2/3 deletion (WHO, 2020). One possibility of the high number of HRP-2 gene deletion detected in this study is the testing pressure of the selected population that participated in evaluation study. Other studies, from African countries (Ethiopia, Mali, Ghana, Tanzania and Uganda) have reported similar finding of presence of HRP-2 gene deletion parasites in the community (Golassa *et al.,* 2020; Koita *et al.,* 2012; Thomson *et al.,* 2019) that lead to false negative mRDT results. Additionally, studies that were conducted in Madagascar, Ethiopia and Kenya to investigate the presence of gene deletion and reported hrp2 gene deletion of 1.4% at Ethiopia samples and 0.6% of Madagascar samples, and dual *P.fhrp2/3* of 2.0% for Ethiopia samples (Rogier *et al.,* 2022) and 10% in western and eastern of Kenya (Beshir *et al.*, 2017). Kaaya *et al.* (2022) reported 1.6 % of P.frp2 and 50% P.frp3

proportion of gene deletion in study conducted in the 2 regions of Tanzania. Even with these findings, still other populations are safe as indicated by other study conducted in Tanzania which did not detect hrp2/hrp3 gene deletion (Bakari *et al.,* 2020). The difference observed from the studies from Tanzania, could be attributed to type of specimen used to analyze gene deletion. Our study used the venous whole blood sample while other studies used blood spots samples which may yield different results due to variations in the amount and quality of DNA (Bakari *et al.,* 2020). Since most of the testing is done by mRDT, there is a possibility of selection pressure to occur and leave the HRP-2 gene deletion parasite populations in the community (Agaba *et al.,* 2019).

Performance of the mRDTs can be influenced by many factors. However, samples collected from patients who were report with malaria and co-infected with other diseases, did not affect the performance of the test kits, One Step Malaria *P.f/P.v* Tri-line and One Step Malaria *P.f* tests.The results showed that, there was no interference in the performance of tested kits with common diseases such as respiratory tract infection, urinary tract infection and HIV that, are prevalent in the malaria endemic areas. This implies that the test kits are sensitive and specific to malaria diseases. Findings from other studies reported false positive in patients with no recent history of malaria but infected with other diseases like hepatitis C, dengue virus, tuberculosis and schistosomiasis and Toxoplasma gondii infections (Maltha *et al.,* 2013; Lee *et al.,* 2014; Grobusch *et al.,* 2003).

This suggests that's heterophile antigens produced by these pathogens resulted in the Immunologic cross-reaction which cause of these false positive results (Haberichter *et al.,* 2017). Furthermore, venous blood collected in different anticoagulants, did not affect the performance of One Step Malaria *P.f/P.v* Tri-line and One Step *P.f* mRDTs kits mRDTs. Studies that evaluated the influence of anticoagulants in mRDTs are scarce. Nevertheless, effect of anticoagulants in detection of malaria by other point of care test, reported no influence of anticoagulant in quantification of the parasitemia (Martin *et al.,* 2017). The result from this study are similar to what has been reported in our study, hence it is performance meets the WHO prequalification assessment criteria and can be used for malaria diagnosis in the endemic areas.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The current study demonstrated that the sensitivity and specificity of novel One Step Malaria *(P.f/P.v)* Tri-line and One Step Malaria *P.f* mRDTs test kits are similar to test kits that are currently in the marker and routinely used in the health care facilities in Tanzania and elsewhere. Despite the limitations mentioned above, the mRTDs kit's performance is acceptable and should be recommended to supplement the existing registered mRDTs and use in malaria diagnosis in endemic areas since have undergone WHO-prequalified assessment and met minimum performance criteria before been acceptable for registration and procured for malaria endemic counties. The summary of the assessment report for all WHO prequalified malaria RDTs are made available to the public to help with the selection of the suitable test kits. There are many malaria rapid diagnostic tests which are used globally, however, the new test kits once approved come to supplement the testing capacity in the countries that still have malaria, but also complement the elimination strategies through improved diagnosis and strengthening malaria surveillance and generating data for response.

5.2 Recommendations

With all the achievement, the reported case of HRP-2 gene deletion is a big threat in the introduction of the improved mRDTs kits. The proportion of samples with HRP-2 gene deletion reported in the study population and causing false negative mRDT results warrant further studies to map the prevalence of gene deletion and determine how it affects the current malaria control strategies.

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RESEARCH OUTPUTS

(i) Publication

Mwangonela, Z. E., Ye, Y., Rachel, Q., Msuya, H. M., Mwamlima, T. G., Mswata, S. S., Chaki, P. P., Kimaro, E. G., Mweya, C. N., Mpina, M. G., & Mwangoka, G. W. (2023). Field evaluation of the novel One Step Malaria Pf and Pf/Pv rapid diagnostic tests and the proportion of HRP-2 gene deletion identified on samples collected in the Pwani region, Tanzania. *Bulletin of the National Research Centre*, *47*(1), 17.

(ii) Poster Presentation