Development of Real-Time TaqMan based Assay for the Diagnosis of Malaria

By

Laleen Saeed

(Registration Number 00000327371)

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Supervisor

Dr. Salik Javed Kakar

Atta Ur Rahman School of Applied Biosciences

National University of Sciences and Technology, Islamabad

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We hereby recommend that the dissertation prepared under our supervision by: Student Name **Laleen Saeed** and Reg No : **00000327371** Titled: "**Development of Real Time Taqman based Assay for the Diagnosis of Malaria**" be accepted in partial fulfillment of the requirements for the award of **MS Degree in Healthcare Biotechnology** degree with (A grade).

Examination Committee Members

1. Name: **Dr. Dilawar Khan** Signature: _____

Dr. Dilawar Khan
Assistant Professor
Atta-ur-Rahman School of Applied Biosciences (ASAB), NUST Islamabad

2. Name: **Dr. Tahir Ahmad** Signature: _____

Dr. Tahir Ahmad
Associate Professor
Industrial Biotechnology
Atta-ur-Rahman School of Applied Biosciences (ASAB), NUST Islamabad

3. Name: **Dr. Malik Badshah Yousafzai** Signature: _____

Supervisor's name: **Dr. Salik Javed Kakar** Signature: _____ Date: 5th Jan, 2023

Associate Professor
DR. SALIK J. KAKAR
MBBS, MSc (UK), PhD (UK)
Department of Healthcare Biotechnology
ASAB, NUST, Islamabad

Dr. Sobia Manzoor
Tenured Professor
Head of Department (HoD)
Deptt. of Healthcare Biotechnology
Atta-ur-Rahman School of Applied Biosciences (ASAB), NUST-Islamabad

Head of Department — Date: 06.01.2023

COUNTERSINGED

Dean/Principal

Date: _____

HESIS ACCEPTANCE CERTIFICATE

is certified that the contents of thesis entitled **Development of Real-Time Taqman based Assay for the Diagnosis of Malaria** has been found satisfactory for the requirement of the degree.

upervisor: _____

Associate Professor
DR. SALIK J. KAKAR
MBBS, MSc (UK), PhD (UK)
Dept. of Healthcare Biotechnology
ASAB, NUST, Islamabad

r. Salik Javed Kakar

tta-ur-Rahman School of Applied Biosciences

ational University of Science and Technology

ead of Department: _____

Dr. Sobia Manzoor
Tenured Professor
Head of Department (HOD)
Dept. of Healthcare Biotechnology
Atta-ur-Rahman School of Applied Biosciences (ASAB), NUST Islamabad
06-01-23

r. Sobia Manzoor

tta-ur-Rahman School of Applied Biosciences

ational University of Science and Technology

rincipal: _____

Hussnain A. Janjua
Principal
Atta-ur-Rahman School of Applied Biosciences (ASAB)
NUST, Islamabad

r. Hussnain Janjua

tta-ur-Rahman School of Applied Biosciences

ational University of Science and Technology

RTIFICATE FOR PLAGARISIM

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Associate Professor
DR. SALIK J. KAKAR
MBBS, MSc (UK), PhD (UK)
Dept of Healthcare Biotechnology
ASAB, NUST, Islamabad

Supervisor

Dr.Salik Javed Kakar

Associate Professor

ASAB, NUST

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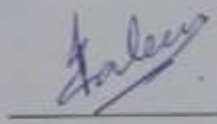

Signature of Student

Laleen Saeed

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Laleen Saeed

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LIST OF ACRONYMS

B

BHQ	Black Hole Quencher

C

CT	cycle threshold
cy5	Cyanine 5
Cy3	Cyanine 3

D

dNTPS	**Deoxynucleotide triphosphates**
℃	degree celcius

F

FAL	Falciparum
FP1	Forward Primer 1
FAM	6’ Flourescein

H

HPLC	High performance liquid chromatography
HEX	Hexachloro-fluorescein
HRP2	Histidine rich protein 2

I

INBs	Insecticide treated beds
IRS	Indoor residual sprays

L

LAMP	Loop mediated isothermal Amplification
LOD	Limit of detection

M

Mal	Malariae
MPH	microtiter plate hybridization method
ml	milliliter

N

NCBI	National Center of Biotechnology Information

O

OVA	Ovale

P

pLDH	parasite's lactate dehydrogenase
P.	Plasmodium
PCR	Polymerase Chain Reaction
Pf	Plasmodium falciparum

R

RT	Real-Time
RBCs	Red Blood cells
ROX	6-carboxyl-X-Rhodamine
RP1	Reverse primer 1
RDT	Rapid diagnostic test

Rpm	revolution per minute
S	
SSUrRNA	small subunit ribosomal RNA
T	
TAMRA	tetramethylrhodamine
Tex red	Texas red
U	
µL	microliter
V	
VIC	Victori

ABSTRACT

Every year Pakistan records 3.5 million suspected and confirmed cases of malaria with *P. vivax* and *P.falciparum* infections being the most common. Even though microscopy is the gold standard for malaria diagnosis, it has poor sensitivity and is time consuming. Microscopy has its limits but molecular detection has overcome these problems by providing higher sensitivity and specificity. To reduce disease burden rapid malaria diagnostic procedures that are highly sensitive and specific for early diagnosis along with differentiation of plasmodium species is essential which is critical for treatment of infection A multiplex Real-Time PCR method is designed for the detection and differentiation of Plasmodium into *P.malariae, P.vivax, P.falciparum and P.ovale* to improve diagnosis and treatment of malaria. One primer set targeting a conserved region of the 18s rRNA gene are designed and this region is variable enough to design four different specie specific TaqMan probes. Real-time PCR detected *Plasmodium vivax* in 21 samples out of 52 blood samples. The assay has sensitivity of 4.1 parasites/μL for *plasmodium vivax*. The developed assay is convenient to perform and results are produced in 1 hour and 30 mins. Therefore, this can provide an alternative method for early diagnosis of malaria and can also be used in epidemiological studies.

1. INTRODUCTION

Plasmodium is a protozoan parasite that causes Malaria. It is a fever illness that is spread through the bites of female Anopheles mosquitoes. *Plasmodium vivax, Plasmodium falciparum, Plasmodium malariae, Plasmodium ovale, Plasmodium knowlesi* are the five different species of plasmodium that are responsible for causing malaria in humans(Mhlanga *et al*., 2022). *Plasmodium falciparum* infections are life threatening among all having the highest mortality rate whereas Plasmodium vivax infections are the most common.(Crutcher & Hoffman, 1996a). The disease is characterized by high grade fever, chills, splenomegaly and anemia.

In the year 2021, 247 million cases of malaria and 619,000 deaths were recorded due to malaria worldwide(*Fact Sheet about Malaria*, n.d.-a). In Pakistan, malaria is endemic and about 60% of its population lives in endemic regions. The number of confirmed and suspected cases of malaria each year are 3.5 million. However, in the year 2022 from January to August, 3.4 million suspected cases were recorded compared in 2021 the suspected cases were 2.6 million. Laboratory testing has identified more than 170,000 cases, of which 77% cases are of *Plasmodium vivax* and 23% cases of *Plasmodium falciparum*, which accounts for serious and fatal cases.

Malaria is clinically diagnosed based on signs and symptoms. As a result of variable and non-specific symptoms that malaria produces, it's diagnosis is still a challenge and cannot be based only on symptoms. An enlarged spleen may also be confused for leishmaniasis, schistosomiasis and typhoid fever. Therefore, at the time of diagnosis the signs and symptoms of malaria should be differentiated from these diseases (Tangpukdee *et al*., 2009a).

Treatment for malaria is determined by the type of parasite that has caused the illness, the location of the infection in the world, and the complexity of the infection. The first two factors help in determining the possibility that the organism is resistant to particular antimalarial medications (Prevention, 2019b). Artemisinin combination therapy (ACT) or chloroquine is used in the treatment of *P. vivax* malaria when the infection has reached the blood stage (Chu & White, 2021). Treatment of falciparum malaria has been made challenging by the broad resistance to chloroquine*(Thu et al.,* 2017).Therefore, quinine, mefloquine, quinidine, pyrimethamine/ sulfadoxine (Fansidar^{R}), artemisinin derivatives (qinghaosu) and halofantrine are prescribed as alternative medications for the treatment of malaria caused by *P. falciparum*. Chloroquine remains extremely effective for treating P. malariae and P. ovale infections. For the eradication of latent forms of *P.ovale* and *P.vivax* in the liver primaquine is effective(Crutcher & Hoffman, 1996b).

Microscopy and rapid diagnostic tests are the diagnostic methods for the identification of plasmodium. Microscopy is regarded as Gold standard for detection of malaria. Method is cheap and produces results fast and does not require complicated machinery. However, it is less sensitive and can detect 50 to 500 parasites/ µL (Feleke *et al*., 2017).

Another diagnostic approach for detection of malarial parasites is Rapid Diagnostic tests. RDT detects specific antigens in the blood by using antibodies through the principle of lateral flow immunochromatography. Most RDTs detects the plasmodium lactate dehydrogenase (pLDH), plasmodium histidine-rich protein II or p-aldolase molecules, which are formed throughout the erythrocytic cycle (HRP2) (Ugah *et al*., 2017). A 100 parasites/ µL threshold is the sensitivity limit for RDTs. False positive results of malaria are produced By RDT because HRP2 remains after an infection has been cleared for several days in the blood (Humar *et al*., 1997). Moreover, false negative results are also produced to gene deletions in HPR2(Berhane *et al*., 2017).

PCR-based approaches for diagnosis of malaria have been used since the late 1980s due to their higher sensitivity, robustness and reproducibility. Therefore, several semi-nested PCR and nested techniques that have been developed over time that enabled the detection of four plasmodium species (Rougemont et al., 2004a). Nested PCR has a sensitivity limit of 5 parasites/ μL. One round multiplex PCR have been developed to overcome the limitations of Conventional PCR procedures(Fitri *et al*., 2022).

The development of quantitative real-time technologies has improved malaria diagnosis. Real-Time PCR offers increased sensitivity (down to 0.02 parasite/μL) simpler operation, and no gel procedures for the identification of PCR products compared to the conventional PCR. Previously, two-step TaqMan based Real-Time PCR assay was designed for detection of *P.vivax, P.falciparum, P.ovale, P.malaria*e with one set of primers that targets the Plasmodium 18s rRNA gene's conserved region. Another study was designed for the detection of *P. knowlesi*. In another study designed for the detection of *P.ovale, P.falciparum* and *P.vivax* making use of three different primer set and probes. Since treatment strategies depend upon the parasite specie causing the infection no study has been designed before detecting and differentiating plasmodium into *P.falciparum, P.malariae, P.ovale* and *P.vivax* using one set of primers targeting a conserved region of 18s rRNA gene and using four different probes in a single reaction.

Therefore, the study aims to develop a qualitative Real-Time TaqMan PCR assay for rapid diagnosis of four malaria parasites (*P. vivax, P. ovale, P.falciparum and P. malariae*)by using one primer set and four different specie specific variable probes in clinical specimens is designed and evaluated in this study .

1.1 OBJECTIVES

1. To Identify a target gene for the diagnosis of malaria and design primer and probes by using online softwares
2. Collection of blood samples from suspected patients of malaria and extraction of their DNA
3. Optimization and Real-Time PCR of the samples
4. Sensitivity and Specificity testing of Real-Time PCR assay

2.LITERATURE REVIEW

2.1. MALARIA

Malaria is a recurring febrile sickness caused by the single-celled Plasmodium also called as "King of Diseases" (Tangpukdee *et al*., 2009b). Malaria is transferred to people through bites of female Anopheles mosquitoes. Five different Plasmodium species causes malaria such as *P. falciparum, P. ovale, P. malariae, P. vivax, P. knowlesi*. The deadliest of all five is *P. falciparum* resulting in higher rates of mortality (Crutcher & Hoffman, 1996a). The clinical symptoms of *P. falciparum* are confusion, neurologic focal signs, severe anemia, coma and respiratory difficulties (Prevention, 2019a). *P. falciparum* infections are common in the African continent. Due to the less clinical severity *P.vivax* is given less attention, however, an increasing number of studies support the evidence that it damages the spleen, lungs and bone marrow causing severe disease. *P. ovale* and *P. Vivax* has similar appearance and remains dormant in the early stages. Therefore, after several years of initial infection re-initiation of the disease can occur. Infections by *P. malariae* is not properly treated, it can also cause chronic illness. In healthy individuals of Western Africa, *P. Ovale* and *P.malariae* causes asymptomatic cases (Lamien-Meda *et al*., 2021a). The mortality rates from *P. knowlesi* are very low but it can also result in severe disease.

2.2. SYMPTOMS OF MALARIA

The initial symptoms of the infection are similar in all species which include flu like symptoms and resemble gastroenteritis, sepsis and other viral diseases (Bartoloni & Zammarchi, 2012). The signs and symptoms may include convulsions, hemolytic anaemia, joint pain, fever, shivering, vomiting, jaundice, haemoglobin in the urine, and headaches(Bartoloni & Zammarchi, 2012).

2.3. STATISTICS OF MALARIA

In the year 2021 worldwide, 247 million cases of malaria and 619,000 deaths were recorded due to malaria. 95 percent cases and 96 percent of deaths are reported from African region. 80 percent deaths are of children under the age of 5 in this region(*Fact Sheet about Malaria*, n.d.-b). Between January and August 2022, Pakistan saw a rise in the number of suspected malaria cases, from 2.6 million to over 3.4 million. The laboratory confirmed more than 170,000 cases, with Plasmodium vivax being the most prevalent specie. In mid-June 2022, following the devastating floods in Balochistan and Sindh provinces had a substantial rise in cases, accounting for 78% of all confirmed cases. However, in Pakistan the number of suspected and confirmed cases of malaria each year are 3.5 million. Pakistan has the highest transmission rate among the six WHO Eastern Mediterranean regions. During the year 2017, KPK reported the highest cases which was 30 percent followed by Sindh 26.5 percent, FATA 21.9 percent, Balochistan, 20.5 percent and least cases reported from Punjab 1.1 percent. The largest number of cases in Pakistan are caused by *P. vivax* contributing to about 81.3 percent whereas *P. falciparum* contributes to 14.7 percent of cases and mixed infections are 4 percent (World Health Organization, 2017b).

2.4. LIFE CYCLE OF PLASMODIUM

Life cycle of malarial plasmodium share similar features across all species of plasmodium. The infection begins when sporozoites are injected into the blood circulation of a person by an Anopheles mosquito. This stage is called the infection stage.

The next stage is the asexual stage which is divided into pre erythrocytic stage and erythrocytic stage. These sporozoites then enter the liver to evade the immune response from the host or enter the lymphatic system. These sporozoites enter the liver sinusoids, the sinusoidal barrier is crossed

and enter the hepatocytes (Tavares *et al*., 2013)(Amino *et al*., 2006). In the hepatocytes they differentiate and begin the first round of asexual replication (Mota *et al*., 2001). Over a range of two to several days multinucleated exo-erythrocytic schizont is formed, which consists of thousands of merozoites. Merozoites are released again into the blood stream in a membrane bound vesicle known as merosome from the liver hepatocytes via the liver sinusoids(Sturm *et al*., 2006). This is end of pre erythrocytic stage of asexual reproduction of malaria. Parasite species such as *P. ovale and P. vivax* enter a latent phase and form hypnozoites instead of schizonts and can result in the re initiation of the infection after several years(Hulden & Hulden, 2011). The erythrocytic stage begins, when merozoites enter the red blood cells. In the red blood cells, first a ring structure is formed that develops into trophozoites. The next stage is the formation of erythrocytic schizont. Each schizont gives rise to new merozoites that are released after the rupture of red blood cells (RBCs) and enter other blood cell resulting in clinical manifestations of the disease. *P. vivax* remains in the RBCs, thereby infecting few RBCs and resulting in low parasitemia. On the other hand, *P. falciparum* do not remain in the RBCs and infects other red blood cells resulting in high parasitemia and is the major reason for causing serious disease(Geleta & Ketema, 2016).

The sexual cycle starts with the formation of gametocytes from a sub population of asexual parasites. During the second blood meal when a mosquito bites an infected person, it takes up these gametocytes. These gametocytes then mature and form male gametocyte called microgametocyte and female gametocyte called mega gametocyte during a process that is called gametogenesis. After reaching the mid gut wall of mosquito, the microgametocyte nuclei undergo division three times to form eight nuclei micro gametes and a single mega gamete(Sologub *et al*., 2011). Each of the microgamete fertilizes a mega gamete and form a zygote. The zygote forms an ookinete and further forms an oocyst after crossing the epithelial layer of mosquito mid gut wall. In the oocyte,

the parasite again undergoes sexual replication to produce sporozoites. This is the end of the sexual stage of reproduction of malaria.

The oocyte ruptures again to release the sporozoites into mosquito cavity. The sporozoites then enter the salivary gland. These sporozoites are present there until the mosquito bites another human and start the cycle again.

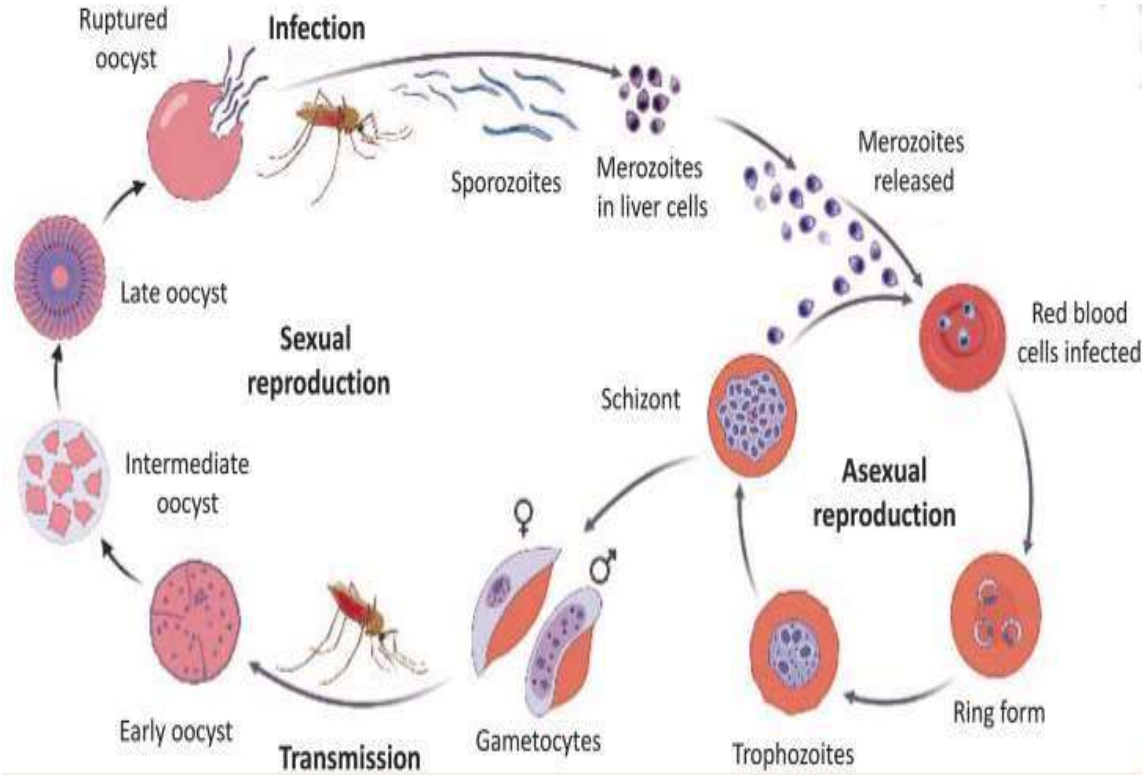


Figure 2.1 Shows life cycle of malaria (Slater *et al*., 2022a)

2.5. PREVENTION OF MALARIA

Malaria can effectively be controlled by vector control strategies which is one of the major component for prevention of disease and reducing transmission rates of malaria (Nyanga *et al*., 2020). Although, no sufficient evidences exist on the effectiveness of the repellents for the prevention of malaria(Maia *et al*., 2018). Insecticide treated beds (INBs) and Indoor residual

sprays (IRS) are used commonly and are effective to prevent malaria and has resulted in decrease in malaria cases in 21st century (Furnival-Adams *et al*., 2021) (Pryce *et al*., 2018) (Pluess *et al*., 2010). As a result of decline in these vector control strategies there is a rise in the transmission rate of malaria, the increase in drug resistance of parasite plasmodium as well as in less cases increase in international travel and migration from countries with cases of malaria has caused a rise in cases of malaria (Pasvol, 2005).

2.6. TREATMENT OF MALARIA

The treatment of malaria differs depending on various factors like the specie of plasmodium causing the infection, disease severity and depending upon where the world infection has been acquired. The treatment of malaria was known long before in 17th century in which quinine was used to relieve the symptoms of malaria fever(Achan *et al*., 2011). Quinine has several harmful effects but it is still used in cases where the parasite has become resistant to the newer drugs or for more severe cases of malaria. Chloroquine is a newer drug which is a combination of mefloquine, sulfadoxin, pyrimethamine, artemisinin and primaquine. Resistance against *P. vivax* and *P. falciparum* was observed by the late 19th century. *P.falciparum* developed resistance against artemisinin in the second decade of 21st century in Southeast Asia(Duru *et al*., 2016). Due to the significant role of artemisinin containing combination therapies against malaria, the resistance is a major source of concern for many.

2.7. DIAGNOSTIC STRATEGIES FOR MALARIA

Proper diagnosis of malaria is required for effective management of the disease. Since malaria has huge global impact in developing countries , as well as in developed countries where accurate

diagnostic strategies for malaria are lacking has produced interest to develop diagnostic strategies for malaria(Bell *et al*., 2005)(Reyburn *et al*., 2007).

Some of the useful characteristics of diagnostic tool include the ability to identify the type of parasite causing infection as well as to detect parasites, quantify the quantity of parasites and detect low levels of parasites. The various diagnostic strategies include microscopy, rapid diagnostic tests and molecular diagnostic methods (Tangpukdee *et al*., 2009a).

2.8. MICROSCOPY

Malaria is diagnosed conventionally by microscopic examination. It is the "Gold Standard" for detection of plasmodium. The technique enables detection of malaria causing parasites as well as to differentiate between different parasite stages and the ability to quantify parasite density (Costa *et al*., 2021a).

2.8.1. METHODS FOR PREPARING MICROSCOPIC SLIDES

Microscopy technique has two variations.

1. Thick Blood Smear
2. Thin Blood Smear

2.8.1.1. THICK BLOOD SMEAR

A thin layer of blood is added on a slide, mixed in a circular motion, and allowed to air dry to create a thick blood film. Giemsa stain is applied to the dry spot for 20 minutes, after which it is rinsed with buffered water for 3 minutes. The glass slide is then allowed to dry before being prepared for microscopic analysis (Tangpukdee *et al*., 2009a).

2.8.1.2. THIN BLOOD SMEAR

Thin blood film is made by dipping the soft side of the spreader in the blood and then placing it on a glass slide at an angle 45 and then quickly spreading the blood on the glass slide. The slide is air dried and then washed with absolute methanol. The slide again dried and fixed with Giemsa stain. The slide is rinsed at the end by dipping it in and out of buffered water. After allowing the slide to dry, it is ready for microscopy (K *et al*., 2006).

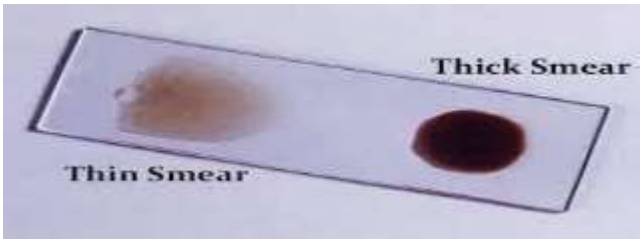


Figure 2.2 Shows thin and thick blood smear glass slide

2.8.2. ADVANTAGES OF MICROSCOPY

Microscopy is cheap (costs about 0.20 dollars per malarial sample), fast (results are generated after 1 hour of sample collection) and does not require complicated instruments(Costa *et al*., 2021b). However, in microscopy the staining and examination process requires trained labour in cases of low parasitemia as well as in detecting mixed malaria infections.

2.8.3. DISADVANTAGES OF MICRSCOPY

Microscopy has low sensitivity for the detection of malaria. An expert microscopist can detect 50 parasites/ µL (Wu *et al*., 2015), whereas an average microscopist detects 50-100 parasites/ µL (Tangpukdee *et al*., 2009a). Microscopy is not reliable in terms of specie identification particularly in case of *P. knowlesi* because it resembles in early ring form with *P.falciparum* and in later stages with *P.malariae* (Amir *et al*., 2018). As result of these shortcomings, results of microscopy need

to be confirmed by another diagnostic techniques in order to increase the accuracy of detection of malaria.

2.9. RAPID DIAGNOSTIC TESTS (RDTs)

Rapid Diagnostic Tests (RDTs) is another way to diagnose malaria. RDTs uses antibodies that detects malaria specific antigens in blood through lateral flow Immunochromatography(World Health Organization, 2017a).

Immunochromatography is based on the capillary flow which combines reagents and separation of molecules by migration on a solid support. It involves antigen antibody reaction for the identification and detection. The immunochromatography tests comprise of nitrocellulose membranes that moves the sample molecules from the application pad through the release pad having lyophilized labelled antibodies along the strip. When an antigen antibody complex is formed, blue line or red purple line is produced, if latex particles or colloidal gold are used as conjugates respectively. Migration along the nitrocellulose membrane is rapid, which results in fast detection of the target antigen(Maltha *et al*., 2013).

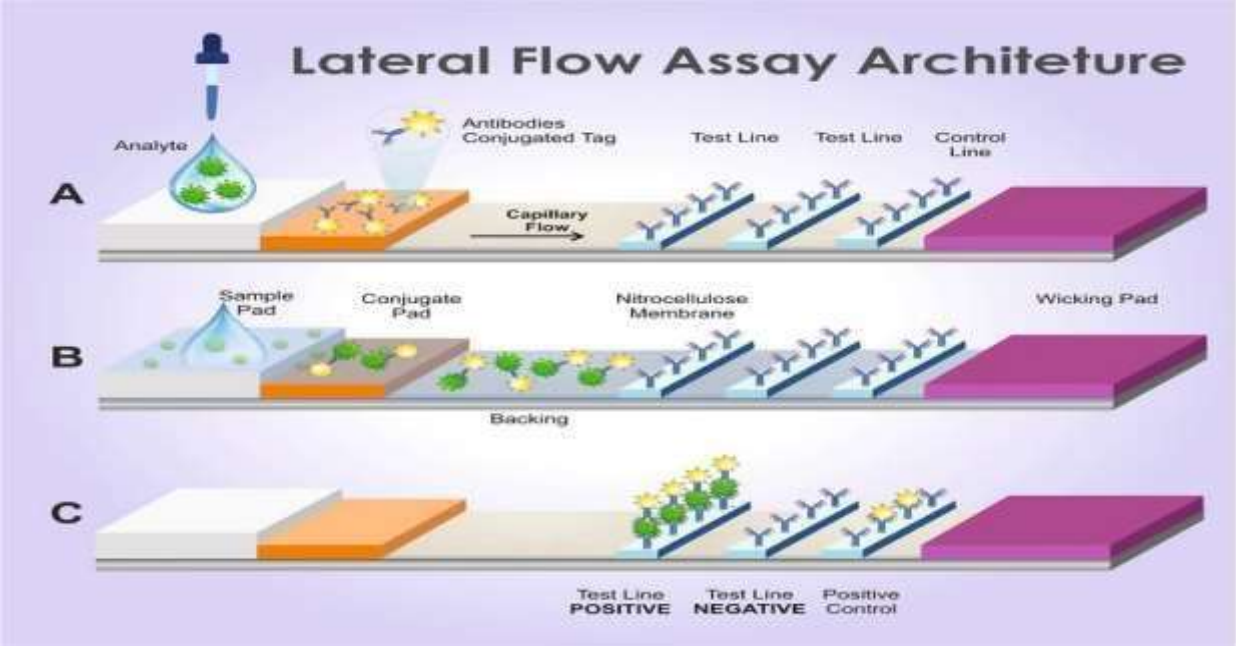


Figure 2.3 Shows the lateral flow architecture in which part A illustrates that through capillary action sample of analyte molecules passes through an antibody-containing nitrocellulose membrane. The reaction between analyte molecules with control line and test line is shown in Part B. A positive result for an analyte found in the sample is shown in Part C(Costa *et al.*, 2021b)

RDTs are developed to diagnose a single specie, multiple species or to discriminate between falciparum and non-falciparum infections. The majority of RDTs are made to detect a water-soluble protein, Histidine Rich Protein 2 (HRP2), generated by *P. falciparum* trophozoites and young gametocytes and are specific to *P. falciparum*. Some designed RDTs react to the *P. vivax*-specific Plasmodium lactate dehydrogenase (pLDH). Other RDTs contain aldolase that are pan specific and is able to detect all species of malaria (Ling *et al*., 2019). Recently, an new rapid test has been develop that detects *P.knowlesi*(McCutchan *et al*., 2008). Currently, 86 rapid diagnostic tests for malaria are available from 28 different manufacturers(Tangpukdee *et al*., 2009a).

2.9.1. RAPID DIAGNOSTIC TESTS ON THE BASIS OF BANDS

RDTs are of three types depending on the number of bands

Two Bands

Three Bands

Four Bands

2.9.1.1. TWO BANDs

Two-band RDTs can detect the Histidine Rich Protein 2 that is specific to *P. falciparum* (Maltha *et al*., 2013).

2.9.1.2. THREE BANDs

Two antigens of *P.falciparum* such as Histidine Rich Protein 2, lactate dehydrogenase along with pan-pLDH or aldolase can be detected by three band(Maltha *et al.*, 2013).

2.9.1.3. FOUR BANDs

In addition to the pan-malaria antigen, four-band RDTs also identify the antigens characteristic of P.falciparum and P.vivax (Maltha *et al*., 2013).

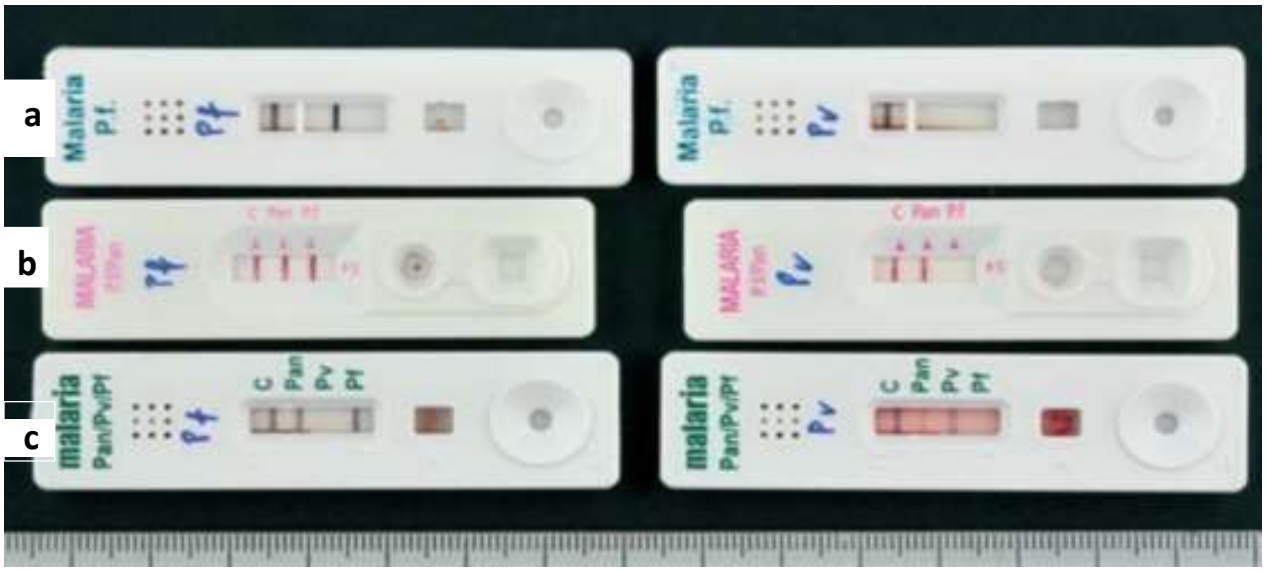


Figure 2.4 Shows Two band, three band and Four Band RDT in the image a, b, c respectively run with *P.falciparum* positive sample on the left and *P.vivax* positive sample on the right. *(Maltha et al*., 2013)

2.9.2. ADVANTAGES OF RDTs

In clinical diagnosis, rapid diagnostic tests have many advantages over microscopy. RDT has shown 90% sensitivity and specificity for *P.falciparum* with a sensitivity of 200 parasites/ µL. RDTs in comparison to other malarial diagnostic tests are simple because they do not require expensive instruments and electricity. RDT per test is inexpensive costing around 0.60 -1.20 dollars. The results of RDT are produced in 15-20 mins (Costa *et al*., 2021b).

2.9.3. DISADVANTAGES OF RDTs

RDT has shown excellent results for diagnosis of malaria, however, some reports have shown variations in the sensitivity of RDTs and are less sensitive than microscopy The sensitivity of RDT falls when the parasitemia decreases below 100 parasites\ µL. RDT also produces false positive

results after treatment as the parasite antigen stays in the blood circulation and can be detected. Since RDTs are usually developed for *P. vivax* and *P. falciparum* and not for *P. malariae* and *P. ovale* therefore, the sensitivity for last two species is the lower. Another limitation of RDT is that due deletions in Histidine Rich Protein 2 and 3, *P. falciparum* infections cannot be detected. RDTs in terms of *P. knowles*i has shown cross reactivity for *P. vivax P. falciparum* limiting the use of RDTs for fast diagnosis. Overall RDT is good diagnostic tool but it should be coupled with other methods for confirmation of malaria (Slater *et al*., 2022a).

2.9.4. RDT IN EDEMIC AREAS

In areas where malaria is endemic and light microscopy is not available such areas may be benefitted from RDT. Therefore, significant improvements need to be made to increase the sensitivity of RDT as wells as the ease of use and affordability. RDT is becoming feasible for rural areas where malaria is endemic and it decrease the time from initial diagnosis to first treatment for cases of imported malaria.

2.10. MOLECULAR DIAGNOSTIC METHODS

Conventional methods for diagnosis of malaria such as Rapid Diagnostic tests and microscopy have several limitations. Diagnostic methods that demonstrate high levels of sensitivity and specificity for fast and accurate diagnosis are required to lessen the disease burden. Developments in molecular biology techniques such as LAMP and PCR have shown tremendous results in detection of low levels of parasite as well as generating new approaches for malaria diagnosis (Slater *et al*., 2022b).

2.11.1 GENES FOR DIAGNOSIS

2.11.1.1. 18S rRNA GENE

Most of these molecular methods target the 18s rRNA small sub unit gene of Plasmodium. This gene has large conserved and variable regions therefore, it can be used for genus specific detection as well as specie specific detection (Chua *et al*., 2015a). Since, small subunit rRNA gene is present in multiple copies it is simple to amplify parasite DNA, making it possible to detect the human malaria parasite species with great sensitivity.

2.11.1.2. COX1 GENE

Another target for PCR based diagnosis is based on cox1 gene. The gene is part of mitochondrial genome of malaria and is 6kb. The mitochondrial genome is derived from female gametocyte and doesn't undergo recombination making it a good target for malarial diagnosis. Moreover, approximately 22 copies of mitochondrial genome are present in plasmodium making it a good target for the specie differentiation(Lamien-Meda et al., 2021b).

Studies have shown that eight times plasmodium species can be identified by molecular methods as compared to microscopy and one third of the infections detected are mixed.

2.12. ISOTHERMAL AMPLIFICATION METHODS

Loop mediated isothermal amplification (LAMP), one of the isothermal amplification techniques, is a fast and less expensive way to diagnose malaria. LAMP utilizes four complex sets of primers that binds to the target region and forms a stem loop secondary structure that act as binding site for other set of primers to attach and continue the amplification process (Becherer *et al*., 2020) (Reboud *et al*., 2019). It basically consists of two steps, the self- elongation step in which

elongation of the template takes place from the stem loop structure that is formed at 3' end and then the elongation step in which the polymerization from the primers actually begins. LAMP is performed by enzyme Bst, from Bacillus stearothermophilus and occurs at 55-65 degrees celcius (Hassan et al., 2022).

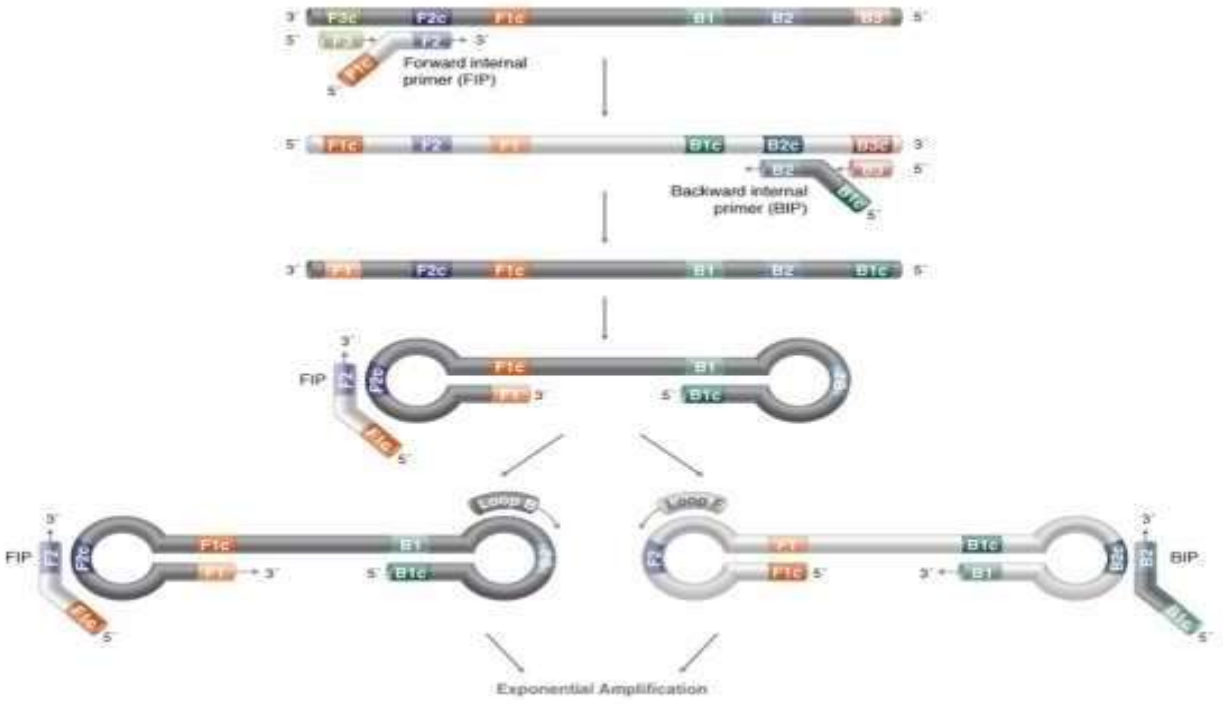


Figure 2.5 schematic representation of loop mediated isothermal amplification mechanism

2.12.1. ADVANTAGES OF LAMP

LAMP detects malaria by targeting the conserved region in P. falciparum 18s rRNA gene (Poon *et al.*, 2006). For P. falciparum as well as P. vivax, P. ovale, and P. malariae , LAMP has demonstrated good specificity and sensitivity (Han *et al.*, 2007) (Aonuma *et al.*, 2008). LAMP

technique for detection of malaria has shown promising results exceeding beyond the sensitivity shown by PCR without the requirement for time consuming and expensive thermal cycling.

2.12.2. DISADVANTAGES OF LAMP

Current LAMP kits lack the ability of multiplexing for different specie detection. Moreover, they produce more false positive results than PCR as well as per sample processing cost is still expensive and the reagents require cold storage. Also, tests need to be performed to check the utility and feasibility of LAMP in clinics(Lamien-Meda *et al*., 2021a).

2.13. POLYMERASE CHAIN REACTION

Molecular methods such as PCR developed by Kary Mullis has been used since 1980s for the diagnosis of malaria. PCR is a laboratory procedure that makes billions of copies of a target region of DNA by using DNA polymerase to amplify a target fragment of DNA. The PCR reaction consists of Taq polymerase, primers, dNTPs and template DNA.

2.13.1 STEPS OF PCR

2.13.1.1. DENATURATION

Initially, the reaction mixture is heated at 96 ℃ to separate the double stranded to form a single stranded template for the following step.

2.13.1.2. ANNEALING

Forward and reverse primer to bind to the complementary sequences in the template when the reaction mixture is cooled down to 55-65℃.

2.13.1.3. EXTENSION

At 72 ℃, Taq polymerase extends the primers forming new strands of DNA.

2.14. CONVENTIONAL PCR

Various molecular biology techniques for detection of malarial parasites have been developed. Such method of diagnosis is also based on targeting the Genus and specie specific sequences that are present in the 18s rRNA gene.

2.14.1. MICROTITER PLATE HYBRIDIZATION

For accurate diagnosis of malaria, a PCR based microtiter plate hybridization method (MPH) has been developed. In the MPH method, the PCR amplifies a fragment of 18S rRNA gene. This is followed by hybridization of probes designed against the specie specific of the gene of the 4 species of plasmodium. The 18s rRNA gene was amplified from blood samples with universal set of biotinylated primers. The PCR product of 138- 150bp size was hybridized with specie specific probes immobilized within microtiter plate well and detected with alkaline phosphatase conjugated streptavidin. Despite being more accurate than microscopy, the microtiter plate hybridization method needs the right set of chemicals for the hybridization plate reader.

2.14.2. NESTED PCR

A technique using nested PCR rather than hybridization was suggested by Snounou et al.,1993. In the first round of PCR, 18s rRNA gene 1.2 kb region is amplified, which is followed by nested PCR using primers that are specie specific. The PCR products from this reaction were detected by a single electrophoretic step. However, this detection method for diagnosis of malarial species is not rapid enough and cannot be applied for routine diagnosis of malaria. The lengthy amplification

(30 cycles) used in the nested PCR could produce false positive resulting from carry-over contamination. Conventional PCR and ethidium bromide method for product is unreliable for exact quantification of the amplification reaction. Moreover, the exact estimation of parasites for large number of samples in a given volume of blood is not practically applicable because precise number of red blood cells and parasites must be obtained which is difficult to be obtained at low parasitemia.

Phenol extraction and ethanol precipitation method for preparation of template DNA for PCR might not be considered appropriate because of the hazardous nature of the reagents used, the time and materials required to prepare the template. Boiling method for preparation template DNA was described that overcome the disadvantages of phenol extraction and ethanol precipitation. In this method, the parasites are boiled to release the DNA. Because DNA is fragmented during the boiling process, DNA longer than 1 kb may be difficult to amplify, leading to false negative results.

2.14.3. ADVANTAGES OF PCR

PCR is highly sensitive technique for detection of malarial parasites as compared to microscopy and RDT. It produces an all or none effect is able to detect as low as 5 parasites\ μL as well as it is also able to differentiate between malarial parasites (Chew *et al.*, 2012).

2.14.4. DISADVANTAGES OF PCR

However, results of nested PCR showed irreproducibility when the samples had parasitemia very low. No specific amplification products are obtained when all the specie specific primers are included in one step reaction from samples that contained DNA from all four malarial parasites. Nested PCR is prone to high rate of contamination because the products of the first reaction are transferred to another tube for the second round of amplification.

2.15. REAL-TIME PCR

Conventional techniques are time consuming and less sensitive. Therefore, the need for an assay that is more sensitive, specific and less time consuming has resulted in the formation of a molecular method for malaria detection consisting of Real-Time PCR. This method has ability to detect low parasite counts, identification of mixed infection as well as to differentiate between different malaria causing species (Mangold *et al.*, 2005).

A molecular biology technique called real-time PCR allows detection and quantitation of the target during each PCR cycle. A reporter molecule generates a fluorescence signal that is used to detect the target. This signal grows with each PCR cycle as the amount of product produced increases.

2.15.1. TYPES OF REAL-TIME PCR

Two types of RT-PCR are developed for the detection of malaria parasites

1. RT- PCR containing SYBR green
2. RT- PCR containing TaqMan Probes

2.15.2. RT-PCR CONTAINING SYBR GREEN

An asymmetric cyanin dye, SYBR green that attaches to any double-stranded DNA's minor groove without any specificity to the sequence. The excitation wavelength of SYBR green is 480nm and the emission wavelength is 520 nm which is comparable to fluorescein. During each extension step, it binds to minor groove of each new copy of double stranded DNA. The bound SYBR green produces a 1000-fold greater fluorescent signal as compared to the unbound dye. As a result of this an increase in fluorescent signal produced that can be observed in Real-Time during the

amplification step and the signal decreases when DNA is denatured. The fluorescent signal should therefore, be measured at the end of elongation step.

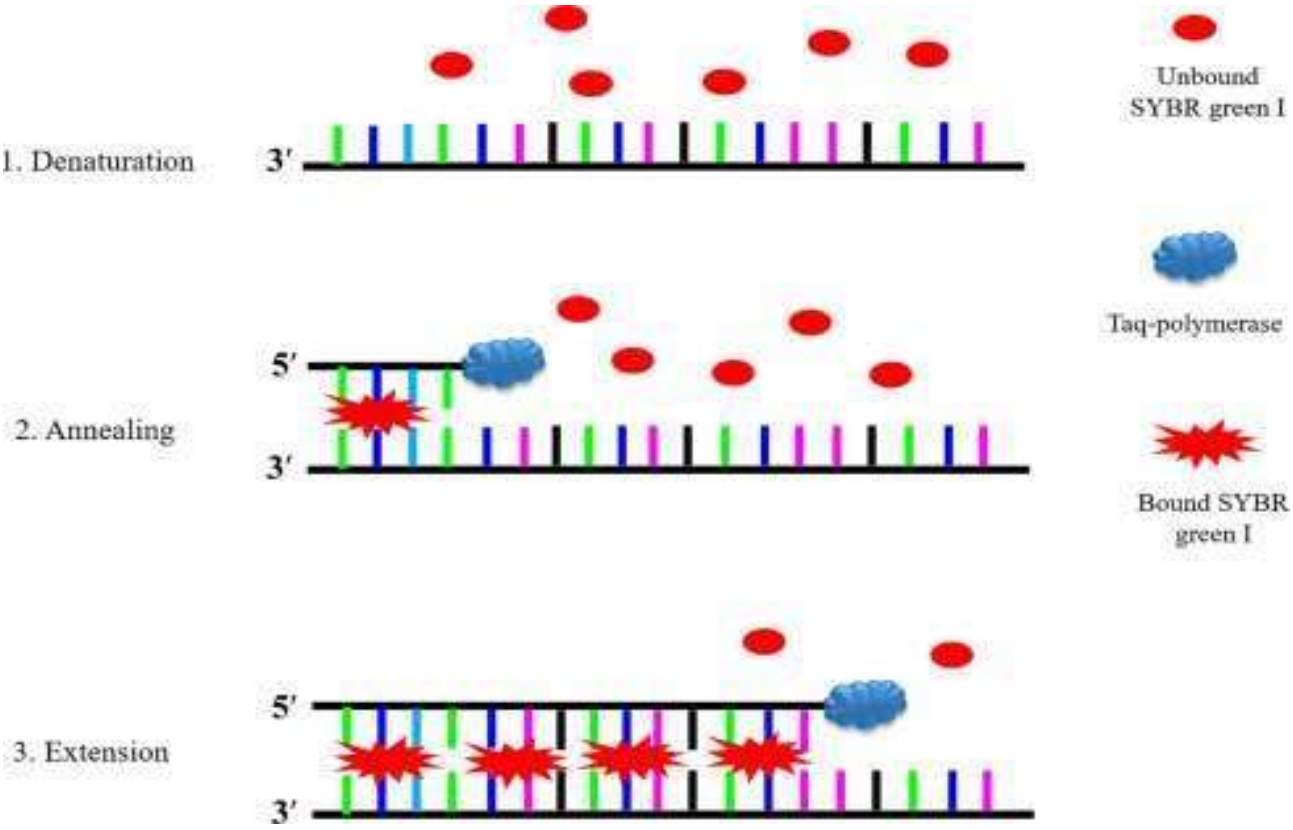


Figure 2.6 shows principle of detection using SYBR green based Real-Time PCR

2.15.2.1 RT-TIME CONTAINING SYBR GREEN FOR MALARIA DETECTION

Real-Time SYBR green PCR method was designed for the identification and discrimination of plasmodium species targeting the Cytochrome b gene *of P. vivax, P. ovale, P.falicparum and P. malariae*. The assay detected 1 parasite/ µL for *P.vivax* and *P. falciparum* and 2 parasites/ µL for *P.malariae* and *P.ovale* (Xu *et al.*, 2015).Another study was designed to detect a high copy number

gene erythrocyte membrane protein 1 in *P.falciparum* in clinically dried blood spot samples of plasmodium. This method detected 9.3 parasites/ mL(Grabias *et al.*, 2019).

2.15.2.2. ADVANTAGES OF RT-TIME CONTAINING SYBR GREEN FOR MALARIA DETECTION

SYBR green based RT-TIME does not require designing an oligo labelled with a fluorescent dye. As a result, primer designing for such type of experimental design is simple as well as the experimental costs are also minimal specifically when detecting many genes that would not require the designing of multiple probes(*SYBR Green I - an Overview | ScienceDirect Topics*, n.d.).

2.15.2.3. DISADVANTAGES OF RT-TIME CONTAINING SYBR GREEN FOR MALARIA DETECTION

SYBR green dye has no specificity to any particular target (Tajadini *et al.*, 2014). This can result in generation of false positive results as it can bind to any double stranded DNA. Target products identification and differentiation requires melting curve analysis.

2.15.3. REAL-TIME PCR CONTAINING TAQMAN

TaqMan based Real-Time PCR works by using the Taq polymerase 5’ to 3’ nuclease activity to detect the products from PCR. The reaction utilizes hydrolysis probe that is conjugated to different dyes that emit fluorescence at different wavelength. The probe is designed complementary to a DNA target region that is present in between the two PCR primers. The probes have higher Tm as compared to the primers so that they should be hybridized to the target region as the primer extension begins.

The 5' and 3' ends of the TaqMan probe are attached a reporter dye and a quencher dye, respectively. Commonly used dyes for reporter are FAM and VIC while for quencher TAMRA is

used. Other reporter dyes include ROX, cy5, cy3 and HEX and quenchers include BHQ2, BHQ3. When the probe is bound to the target DNA, the two fluorochromes are present in close proximity to one another, no fluorescence is emitted as the emission from reporter is suppressed by quencher molecule. During extension step, the Taq polymerase begins to elongates the primers. The Taq polymerase then utilizes its 5, to 3' exonuclease activity and will start to degrade the probe. As a result, the reporter dye molecule is released from the probe, moves away from the quencher, and begins to emit fluorescent signals indicating the presence of the target sequence.

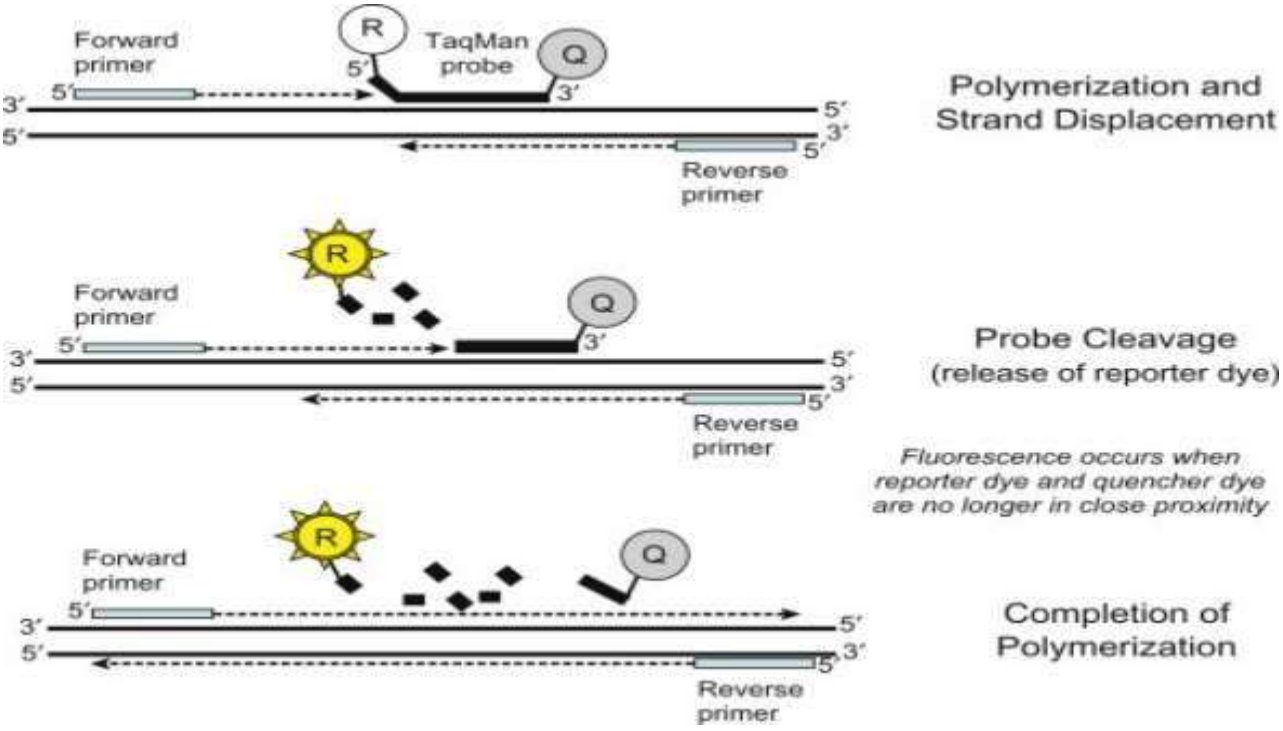


Figure 2.7 shows principle of detection using TaqMan based Real-Time PCR

2.15.3.1. RT-TIME CONTAINING TAQMAN FOR MALARIA DETECTION

To improve the diagnosis of malaria a multiplex RT-PCR was developed. Generic primers were designed targeting a highly conserved region of 18s rRNA gene. Four different probes were designed for detection of *P. ovale, P.falciparum, P.malariae* and *P. vivax* within the primer set region. One copy was detected with RT-TIME using specie specific TaqMan probes(Rougemont et al., 2004b). Another TaqMan based RT-TIME showed high sensitivity of 99.6% and specificity of 100% for detection of vivax malaria (Kim *et al*., 2014). Similarly, RT-TIME based High Resolution Melting Curve method to identify all species of plasmodium and coupled with TaqMan probe designed to differentiate specifically *P. falciparum* from other species. The assay was able to detect 1-2.11 parasites/ µL. The TaqMan probe associated with HRM RT-PCR was able to confirm all *P. falciparum* infections(Lamien-Meda *et al.,* 2021a). RT-TIME assay was developed to detect *P.knowlesi* specie in clinical samples. In this study, previously described studies for the detection of plasmodium species were exploited to design specific probe for *P. knowlesi*. The sensitivity of the assay was 10 parasites/ µL. The assay did not show any cross reactivity with any plasmodium species(Divis *et al*., 2010). Another study was designed to detect *P.vivax, P.falciparum, P.ovale* in whole blood specimens suspected for malaria. The study utilized three sets of primers along with its probes. This Real-Time TaqMan based PCR had a sensitivity of 1.5, 4 and 0.7 parasites / µL for *P.ovale ,P.vivax and P.falciparum* respectively. No cross reactivity was observed with DNA of Toxoplasma gondii and Leishmania infantum(Perandin *et al*., 2004a).

3.METHODOLOGY

3.1. *IN SILICO* METHODOLOGY

3.1.1. TARGET SELECTION

18s rRNA gene was selected for the detection of malaria parasites. 18s rRNA gene sequences *of P. ovale, P. vivax, P.falciparum and P. malariae* were downloaded from NCBI and were used for primer and probes designing.

Table 3.1 shows the accession number of *P.ovale, P. malariae , P.falciparum and P.vivax*

SPECIE	ACCESSION NUMBER
P. ovale	L48987.1
P. malariae	M54897.1
P. falciparum	M19172.1
P.vivax	X13926.1

3.1.2. ALIGNMENT OF GENE SEQUENCES OF 18s rRNA GENE

18s rRNA gene sequences of all four species of Plasmodium were aligned by using Clustal W. After alignment the specie specific and conserved regions of 18s rRNA gene were identified.

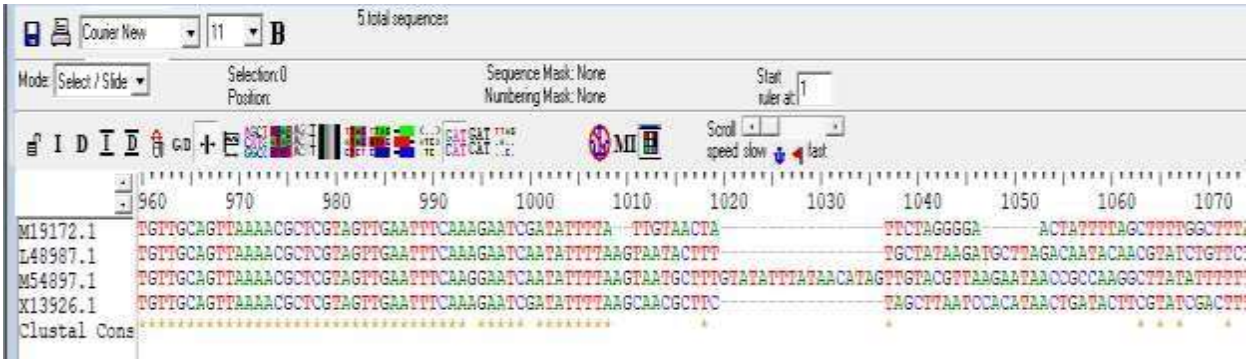
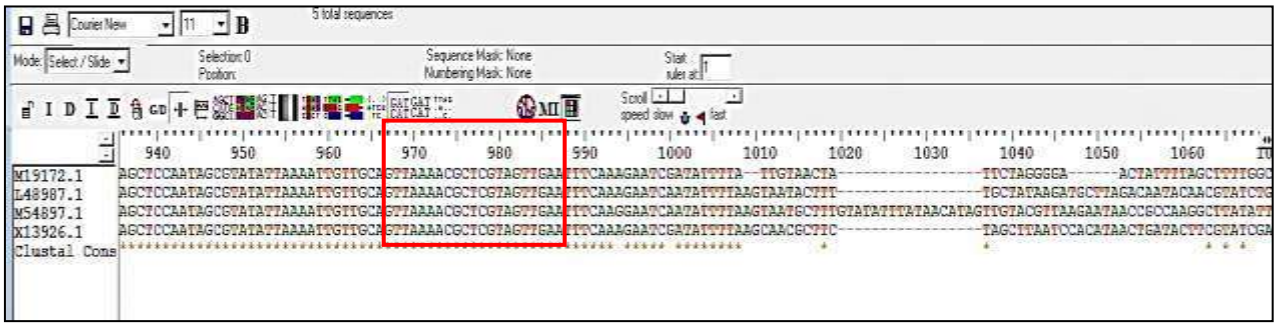


Figure 3.1 shows the conserved regions among all four plasmodium species represented steric whereas variable regions are not represented by steric signs

3.1.3. DESIGNING OF THE PRIMERS AND PROBES

After identification of the conversed and the variable specie specific regions a single set of primer containing forward and reserve primer were designed by manually selected a conserved region among *P. vivax, P. ovale, P. malariae and P.falciparum.*



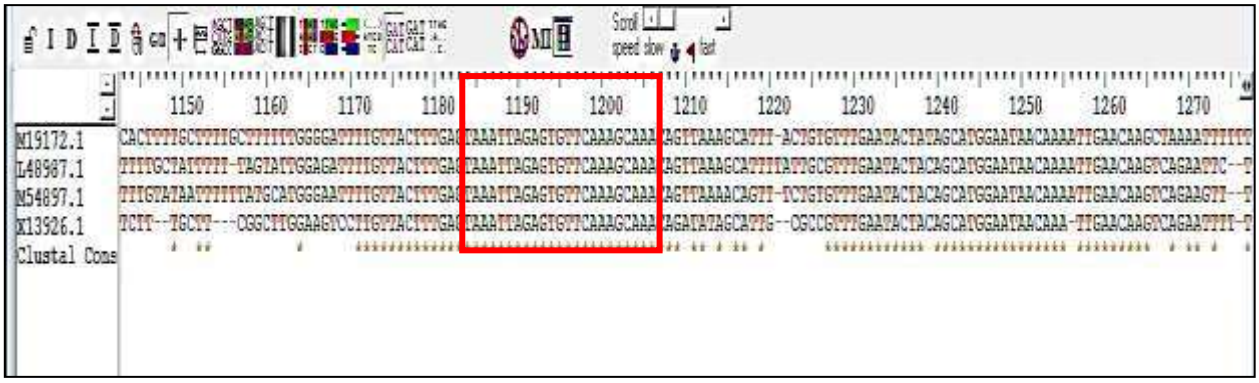


Figure 3.2 shows the conserved regions selected for the designing of primers in red box

Table 3.2 shows primer sequences designed against *P. ovale, P. falciparum, P. malariae,* and *P. vivax* conserved region of 18s rRNA.

Primers	Sequence	Location
Forward (FP1)	5'CAGTTAAAACGCTCGTAGTT 3'	967-987
Reverse (RP1)	5'GCTTTGAACACTCTAATTTACTC 3	1184-1206

The region selected for primer designing was variable enough to enable the designing of four different specie specific probes. Compatible reporter and quencher sequences were added to the 5' and 3' ends of the probes. The Primers and Probes were synthesized by NovoPro Bioscience Inc And purified by Standard Desalting and HPLC respectively

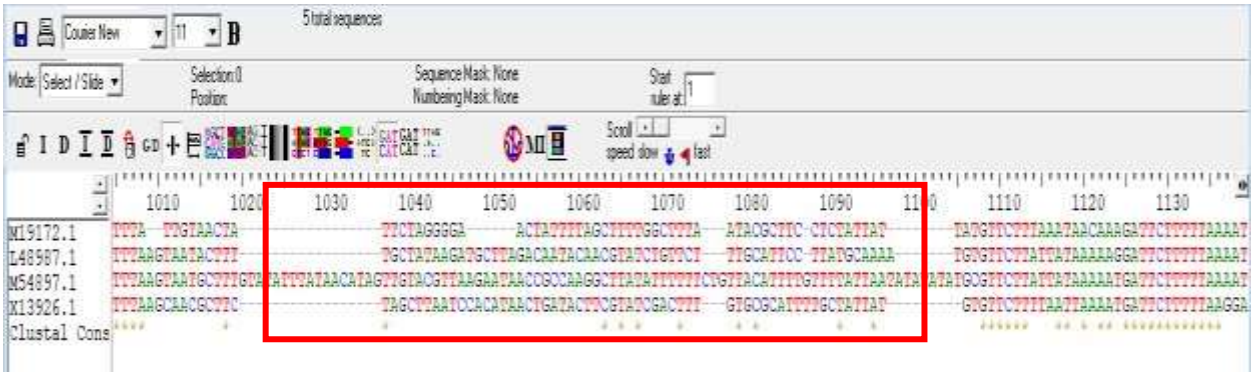


Figure 3.3 shows the variable region among all plasmodium species that was selected for designing Probes

Table 3.3 Shows probes and its sequences designed against the variable region within the region of primer set

Probes	Sequences	Location
P.falciparum	5'FAM TTAGCTTTTGGCTTTAATACGCTTCCTCTA 3' BHQ1	1061-1094
P.ovale	5'Texred AACGTATCTGTTCTTTGCATTCCTTAT GCA 3'BHQ2	1063-1096
P.malariae	5'Cy5 CATAGTTGTACGTTAAGAATAA CCGCCAAGG 3' BHQ2	1034-1064
P.vivax	5' HEX CGT ATC GAC TTT GTG CGC ATT TTG CTA TTA 3'BHQ2	1065-1097

3.1.4. OLIGO CAL

The sequences of primer sets and four probes were added separately in the software to check the formation of potential hairpins, complementary and self-annealing sites in the primers and probes. The GC content, melting temperatures of the primer set and the probes was also determined using OLIGO Cal.

3.1.5. MULIPLEX PCR CHECK

The primers and probes sequences were added in the Thermo fisher primer dimer check to determine whether there is any dimer formation between the primer- primer, primer- probe and probe- probe.

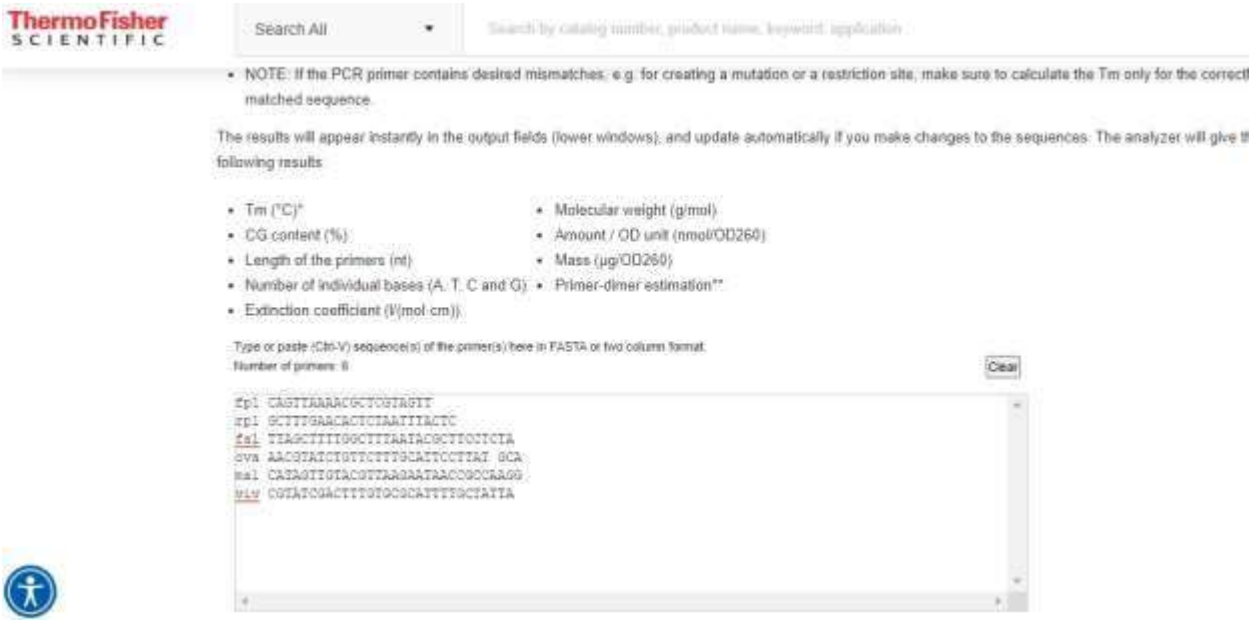


Figure 3.4 Shows that primers and probes sequences added into thermofisher primer dimer Check

3.1.6. INSILICO PCRs

Insilico PCRs of primer set were run for all the four species using SNAP GENE software and the ability of primer set to bind and amplify a conserved region of 18s rRNA gene of all four-plasmodium specie was determined. Each plasmodium specie FASTA sequence previously downloaded from NCBI was uploaded into SNAP GENE and then *Insilico* PCR was run for each specie with a set of forward primer (CAGTTAAAACGCTCGTAGTT) and reverse primer (GCTTTGAACACTCTAATTTACTC).

3.2. *IN VITRO* METHODOLGY

3.2.1. SPECIMEN COLLECTION

A total of 52 microscopically positive whole blood specimens were collected from August to November 2022 from different hospitals of Pakistan. Aliquotes of these blood samples were stored at -20 ℃ to extract Plasmodial DNA for Real-Time qPCR assay.

3.2.2. EXTRACTION OF DNA FROM SPECIMEN

DNA templates for PCR were prepared by extracting DNA from Blood in EDTA tubes using SYSTAAQ Purification Kit. The following protocol was used for extraction of DNA. 400 µL of lysis buffer, 300 µL of blood and 10 µL of carrier RNA was added into the Eppendorf tube. After being vortexed for roughly ten seconds, it was allowed to incubate for ten minutes. Following this, 300 µL of binding buffer was added to the same eppendorf and mixed using a vertex. For 30 seconds, the Eppendorf tube was centrifuged at 13000 rpm. Following this, 700 µL of the aforementioned mixture was put into a spin column and centrifuged for 1 minute at 13000 rpm. The collection tube's filterate was discarded. The spin column was then infused with 500 µL of wash buffer, and it was centrifuged for one minute at 13000 rpm. The washing process was repeated. After that, the spin column was put into an Eppendorf tube, 60 µL of elution buffer was added, and the spin column was centrifuged for 1 minute at 13000 rpm. DNA that has been extracted was kept at -20 °C.

3.2.3. DNA QUALITY AND QUANTITY CHECK

Using Nanodrop 2000 (Thermoscientific, USA), the DNA was measured, and the 260/280 ratio was examined to validate its purity. 260/280 of 1.7- 1.9 is considered good for DNA.

3.2.4. GRADIENT PCR

For primer optimization, a clinically positive sample was processed in order to set the annealing temperature by using gradient PCR. The Gradient mode was set in AB Quant Gene Automated Real-Time PCR System. Gradient PCR profile is as follow. A starting step of 95 ℃ for 5 min, and 30 cycles of 94℃ for 20sec, annealing step with temperature ranging from 46-53 ℃for 30 sec and an extension step 72 ℃ for 45 secs.

Table 3.4 Shows annealing temperatures range set for gradient PCR

GRADIENT TEMPERATURE						
46℃	**47℃**	**48.3℃**	**49.5℃**	**50.5℃**	**51.8℃**	**53℃**

3.2.5. REAL-TIME PCR ASSAY

For detection of *Plasmodium vivax* in 52 clinical samples, RT-TIME was carried out using the AB Quant Gene Automated Real-Time PCR System. PCR experiment reaction mix consists of Forward and Reverse Primer, vivax probe, qScript PCR Mix, and nuclease-free water and template DNA. The reaction was performed in duplicates for each sample.

Table 3.5 shows the components for a 20-ul reaction for the Real-Time PCR Assay

Components	Quantity
Template DNA	5 µL
(FP1)	0.5 µL
(RP1)	0.5 µL
VIV probe	0.5 µL
qScript PCR master mix	10 µL
Nuclease Free water	3.5 µL

The DNA templates were subjected to the following PCR conditions. A starting step of 95 ℃for 5 min, and 30 cycles of 94℃ for 20 secs, 53 ℃for 30 secs and 72℃ for 45 secs. Each reaction was performed in duplicates. A sample was detected positive by its cycle threshold (Ct) value at which emission of the normalized dye reporter was above the background florescence.

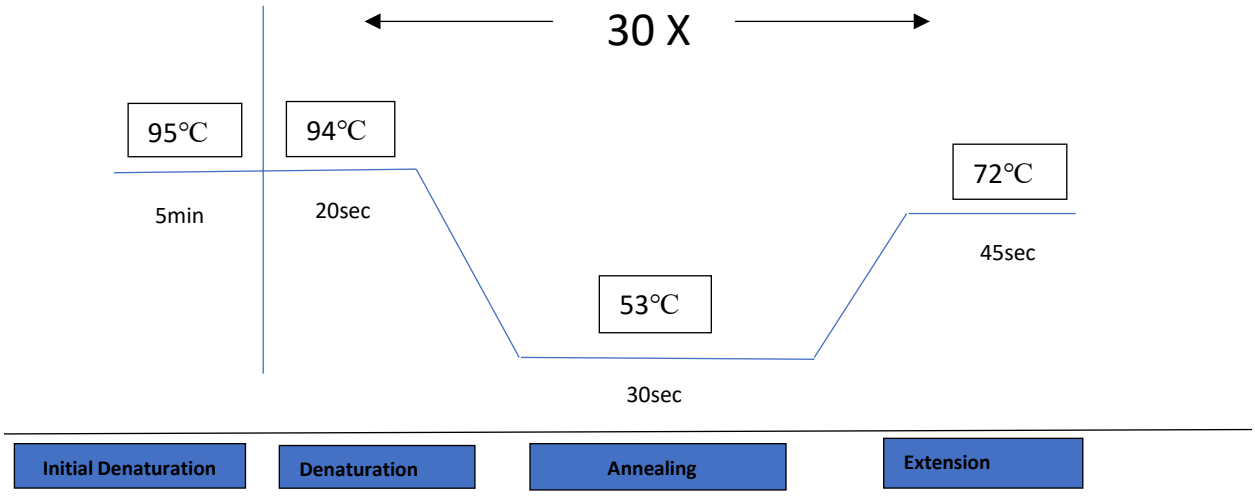


Figure 3.5 shows the thermal cycling profile for *Plasmodium vivax*

3.2.6. SENSITIVITY TESTING OF REAL-TIME PCR

Giemsa-stained thin blood smears were produced and examined under oil immersion (100 magnification) to determine the parasite count of *P. vivax* in the blood specimen. An experienced microscopist evaluated the parasite population against more than 200 white blood cells. Moreover, by thin blood smears parasite densities of P. vivax were also determined. The parasite counts were done against 200 white blood cells, and the parasite density was measured in parasites per microlitre (p/uL) and calculated using the assumption that there are 8000 white blood cells per micro litre of blood.

By using the following formula the parasite density was calculated

$$\text{Parasite density/ } \mu L = \frac{\text{No. of Parasites}}{\text{White blood cells counted}} \times \text{White Blood cells}$$

Whereas, the % parasitemia was estimated by the formula;

$$\%\ \text{Parasitemia} = \frac{\text{No. of Parasites}}{\text{Total no. White blood cells}} \times 100$$

After estimation of the parasite count DNA was extracted from the blood sample. The extracted DNA was further 10- fold serially diluted to contain at least 1 Parasite/ul. The PCR reaction was further performed for each dilution in triplicates to estimate the assay Limit of Detection. A standard curve was also generated to calculate the PCR efficiency.

The following formula is used to calculate the efficiency of the assay

$$\text{Efficiency} = -1+10\ (-1/\text{slope})$$

Serial dilution

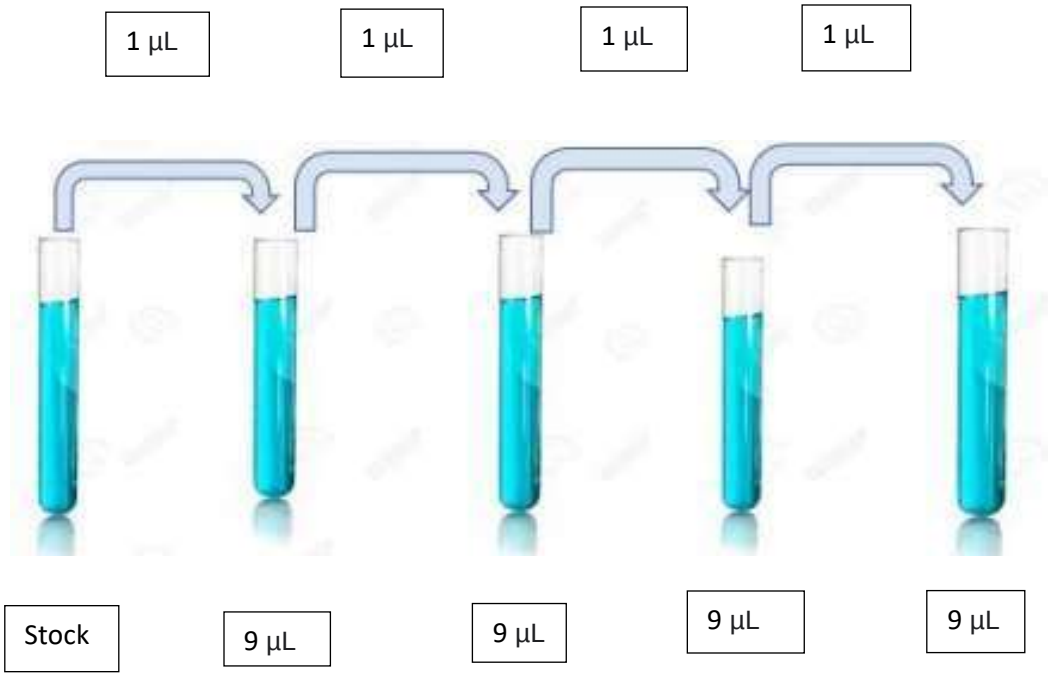


Figure 3.6 shows the principle of 10-fold serial dilution of the extracted DNA with estimated Parasite count

3.2.7. SPECIFICITY ASSAY OF REAL-TIME qPCR ASSAY

In order to calculate the specificity of primers and VIV probe for the detection of *Plasmodium vivax*, Real-Time PCR was performed with the DNA from Human blood *and Salmonella typhi* positive culture broth. Real-Time PCR was performed with the DNA of *Salmonella typh*i using primers and probes for the detection of salmonella. Similarly, Human DNA and Salmonella typhi DNA was also tested using FP1 and RP1 along with VIV probe to determine the specificity of the current method. The reaction was performed in triplicates for both human DNA and *Salmonella typhi* DNA.

4.RESULTS

4.1. INSILICO RESULTS

4.1.1. OLIGO CAL

The results of Oligo Cal for **FP1** 5'CAGTTAAAACGCTCGTAGTT 3', **RP1** 5'GCTTTGAACACTCTAATTTACTC 3' and the four specific Probes **Fal**5'TTAGCTTTTGGCTTTAATACGCTTCCTCTA3',**Ova**5'AACGTATCTGTTCTTTGC ATTCCTTATGCA3',**Mal**5'CATAGTTGTACGTTAAGAATAACCGCCAAGG3',**Viv**5'CG TATCGACTTTGTGCGCATTTTGCTATTA 3' showed that there are no chances of formation of hairpins , self-complementarity and no self-annealing sites in the primer set and the probes.

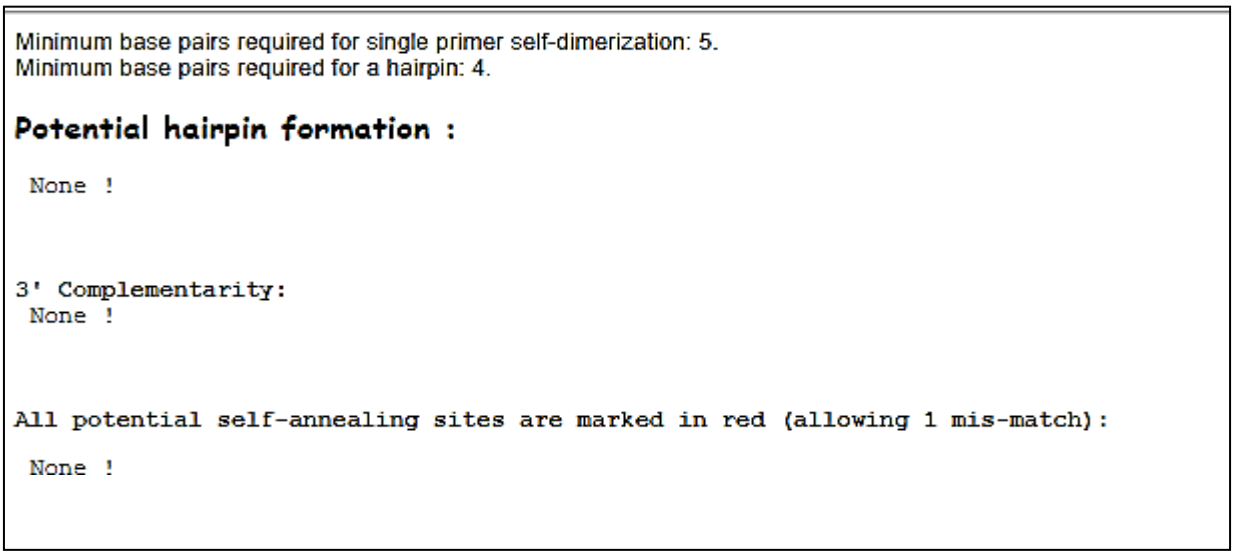


Figure 4.1 Shows that no hairpin formation occurs in primer sets and probes

The GC content, melting temperatures of the primer set and the probes were also determined using OLIGO Cal.

Table 4.1 showing the GC content and Tm of primers and probes determined by Oligo Cal**.**

Primer and Probes	Sequences	GC content	Tm
FP 1	5'CAGTTAAAACGCTCGTAGTT 3'	40 %	47℃
RP 1	5'GCTTTGAACACTCTAATTTACTC 3'	35 %	49℃
P.falciparum	5'FAM TTAGCTTTTGGCTTTAATACGCTTCCTCTA 3' BHQ1	37 %	57.5℃
P.ovale	5'Texred AACGTATCTGTTCTTTGCATTCCTTATGCA3'BHQ2	37 %	57.5℃
P.malariae	5'Cy5 CATAGTTGTACGTTAAGAATAA CCGCCAAGG 3'BHQ2	42 %	60.4℃
P.vivax	5' HEX CGTATCGACTTTGTGCGCATTTTGCTATTA 3'BHQ2	40 %	58.9℃

4.1.2. MULTIPLEX PCR CHECK

The multiplex primer dimer check results have confirmed that there no self-dimers and cross dimers in Forward Primer 5'CAGTTAAAACGCTCGTAGTT 3', Reverse Primer5'GCTTTGAACACTCTAATTTACTC3'**Fal**5'TTAGCTTTTGGCTTTAATACGCTT CCTCTA3',**Ova**5'AACGTATCTGTTCTTTGCATTCCTTATGCA3',**Mal**5'CATAGTTGTA CGTTAAGAATAACCGCCAAGG3',**Viv**5'CGTATCGACTTTGTGCGCATTTTGCTA TTA3'.

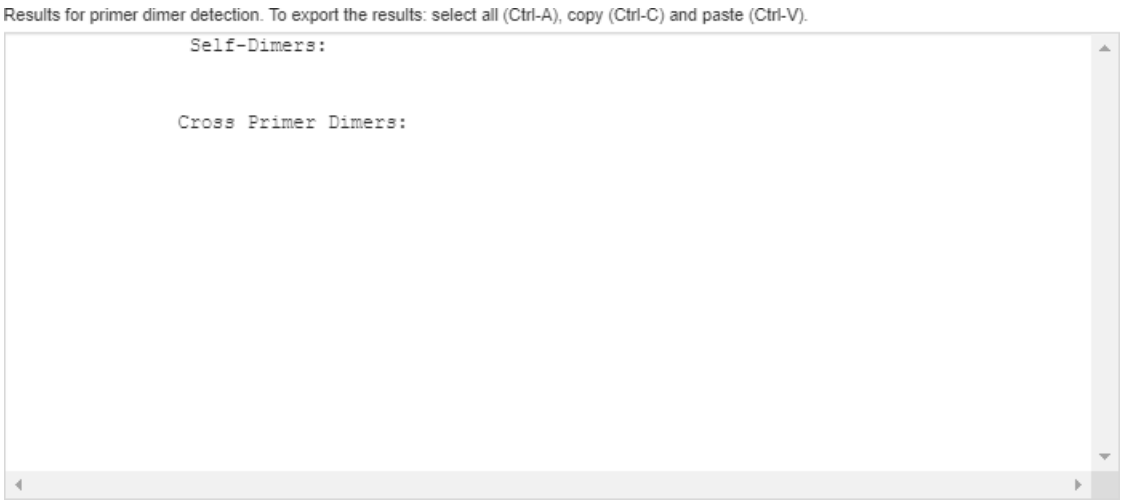


Figure 4.2 shows that no self-dimers and cross dimers formation will form between the primers and probes

4.1.3. INSILICO PCR

The Insilico PCR results generated by Snap Gene depicts that forward primer (FP1) and the reverse primer (RP1) binds to the conserved region of *P.ovale , P.malariae,P. falciparum* and *P.vivax*. The amplication product *of P.falciparum, P.ovale , P.malariae* and *P.vivax* are 202bps, 210bps, 239bps and 205bps respectively Moreover, by using Snap gene it was confirmed that the specie specific probes i-e Fal , Ova , Mal, Viv binds specifically to their own target and does not bind with the 18s rRNA gene sequence of any other specie of plasmodium.

4.1.3.1. INSILICO PCR of *P.falciparum*

The results of *Insilico* PCR of *P.falciparum*'s 18s rRNA gene are represented in the figure below.

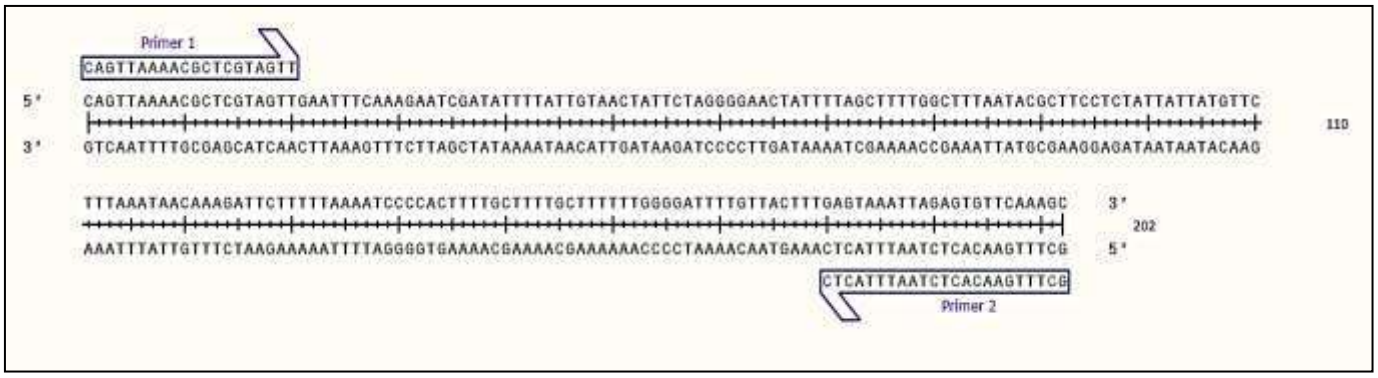


Figure 4.3 shows the amplification product of *P.falciparum* is 202bps

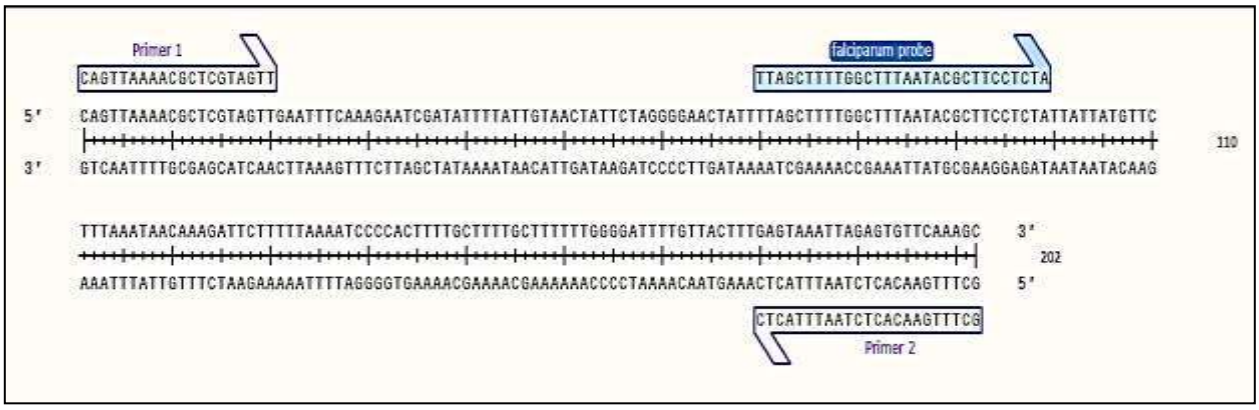


Figure 4.4 shows the binding of falciparum probe to the target region

4.1.3.2. INSILICO PCR of *P.ovale*

The results of Insilico PCR of *P.ovale's* 18s rRNA gene are represented in the figure below.

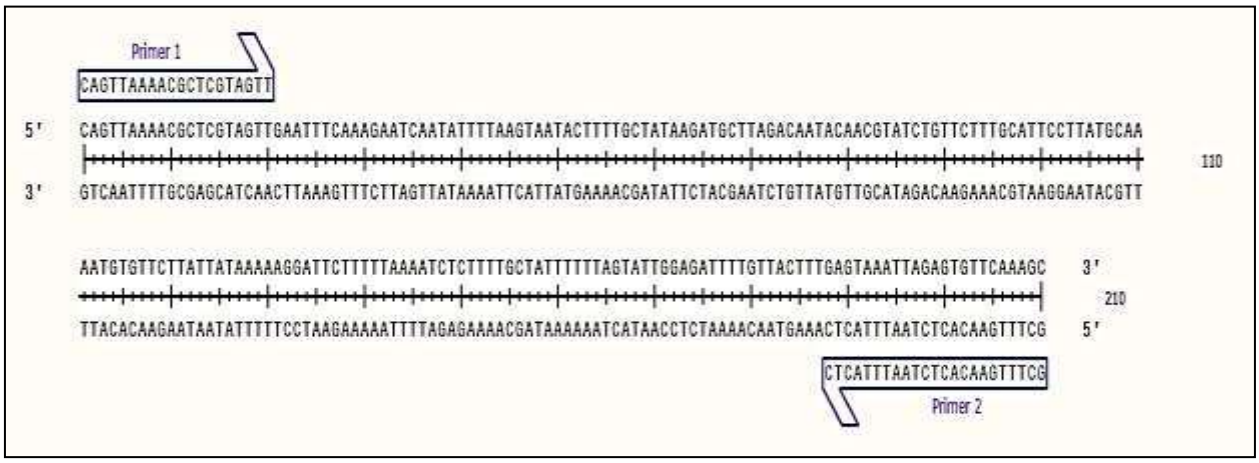


Figure 4.5 shows the amplification product of *P.ovale* is 210bps

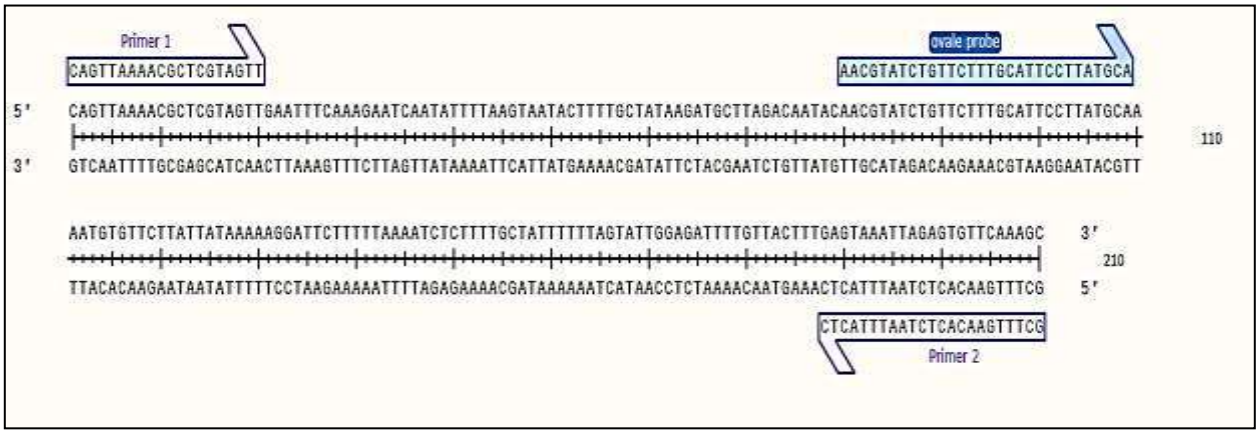


Figure 4.6 shows the binding of ovale probe to the target region

4.1.3.3. INSILICO PCR of *P.malariae*

The results of *insilico* PCR of *P.malariae* 18s rRNA gene are represented in the figure below.

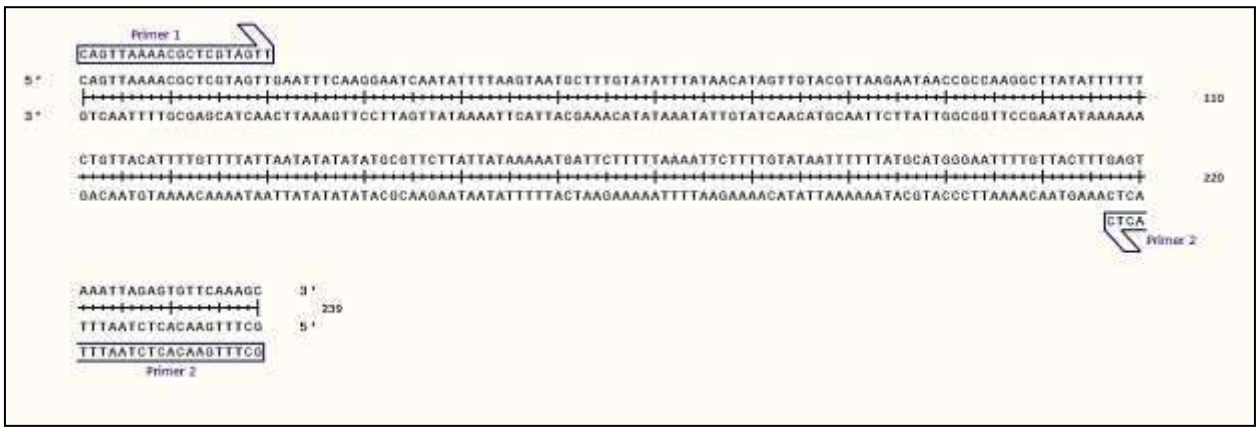


Figure 4.7 shows amplification product of *P. malaria* is 229bps

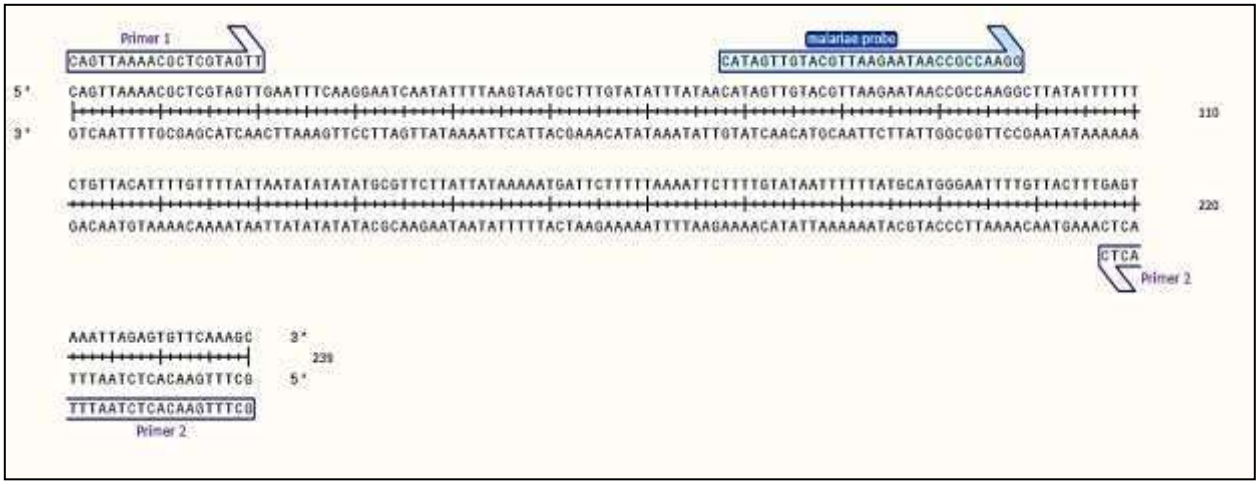


Figure 4.8 shows the binding of *P. malariae* probe to the target region

4.3.1.4. *INSILICO* PCR of *P. vivax*

The results of *insilico* PCR of *P.falciparum* are represented in the figure below.

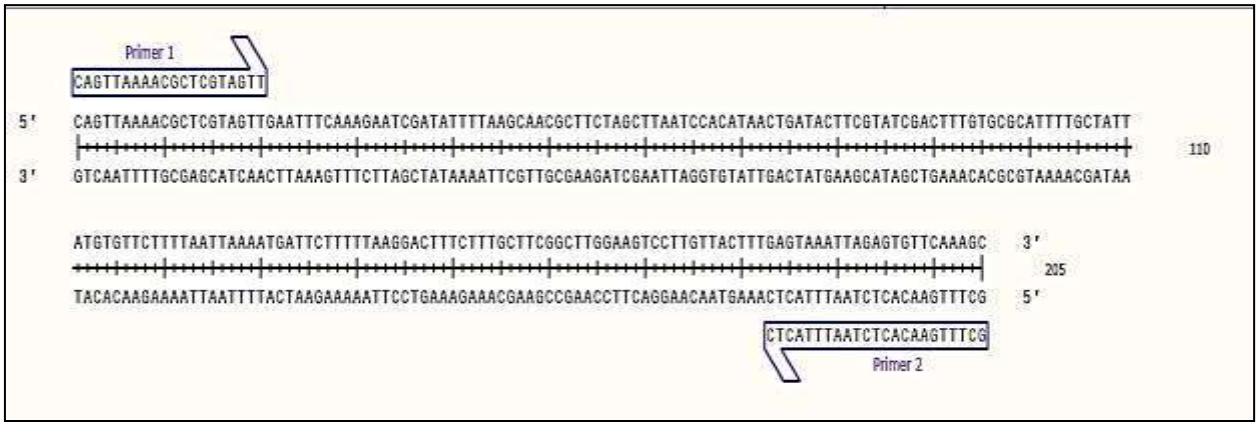


Figure 4.9 shows the amplification product of *P. vivax* is 205bps

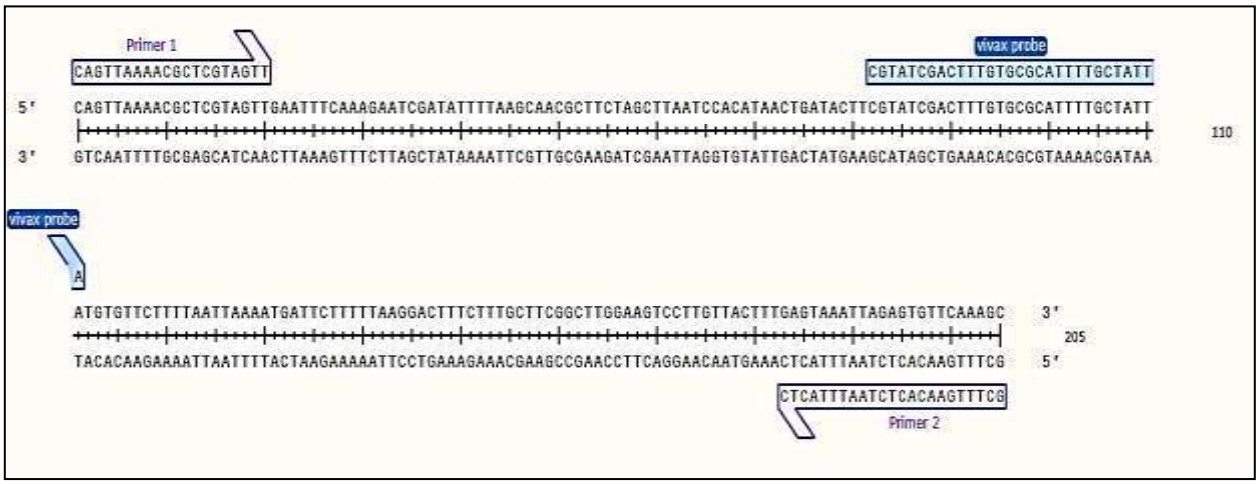


Figure 4.10 shows the binding of vivax probe to the target region

4.2. INVITRO RESULTS

4.2.1. GRADIENT PCR

FP1 5'CAGTTAAAACGCTCGTAGTT 3', **RP 1** 5'GCTTTGAACACTCTAATTTACTC 3' and the vivax Probe 5'CGTATCGACTTTGTGCGCATTTTGCTATTA 3'. Gradient PCR showed that at temperatures 46℃, 47℃,48.3℃,49.5℃,50.5℃ and 53.0℃ primer set and the vivax probe were able to detect the plasmodium Vivax DNA and can be selected as the annealing temperature of the primers and the vivax probe whereas no detection of plasmodium vivax occurred at temperature 51.8℃. Similarly, no fluorescence was detected in the no template control (NTC). Highest temperature 53℃ was selected as the annealing temperature of the primer set.

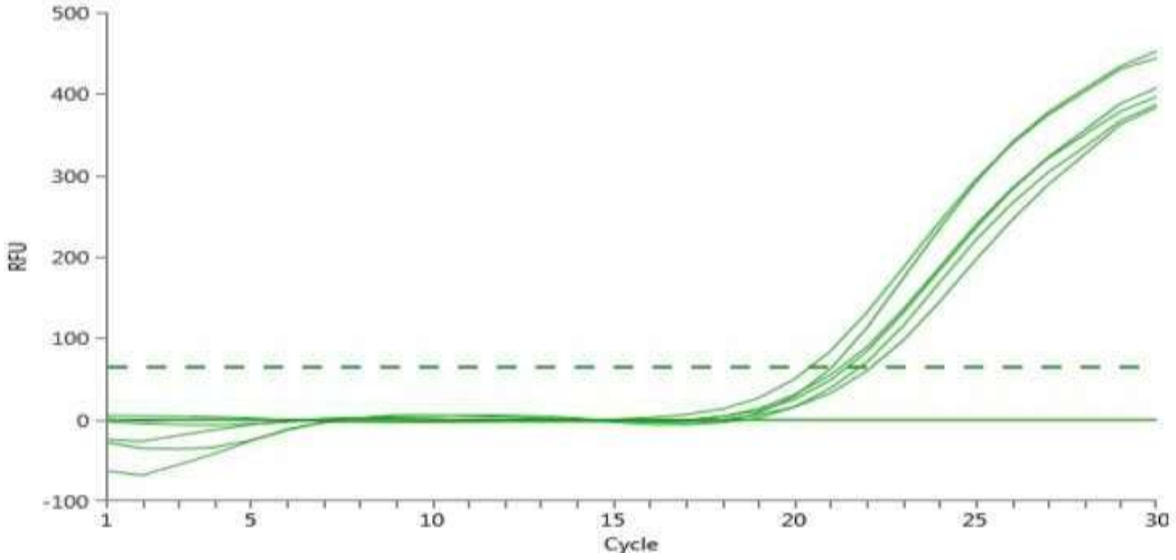


Figure 4.11 shows the temperature gradient Real-Time PCR amplification curve

4.2.2. RT- PCR OF THE SAMPLES

The RT- PCR assay successfully detected *Plasmodium vivax* in 21 samples out of 52 samples that were plasmodium vivax positive by microscopy. No amplification was detected in the no template control.

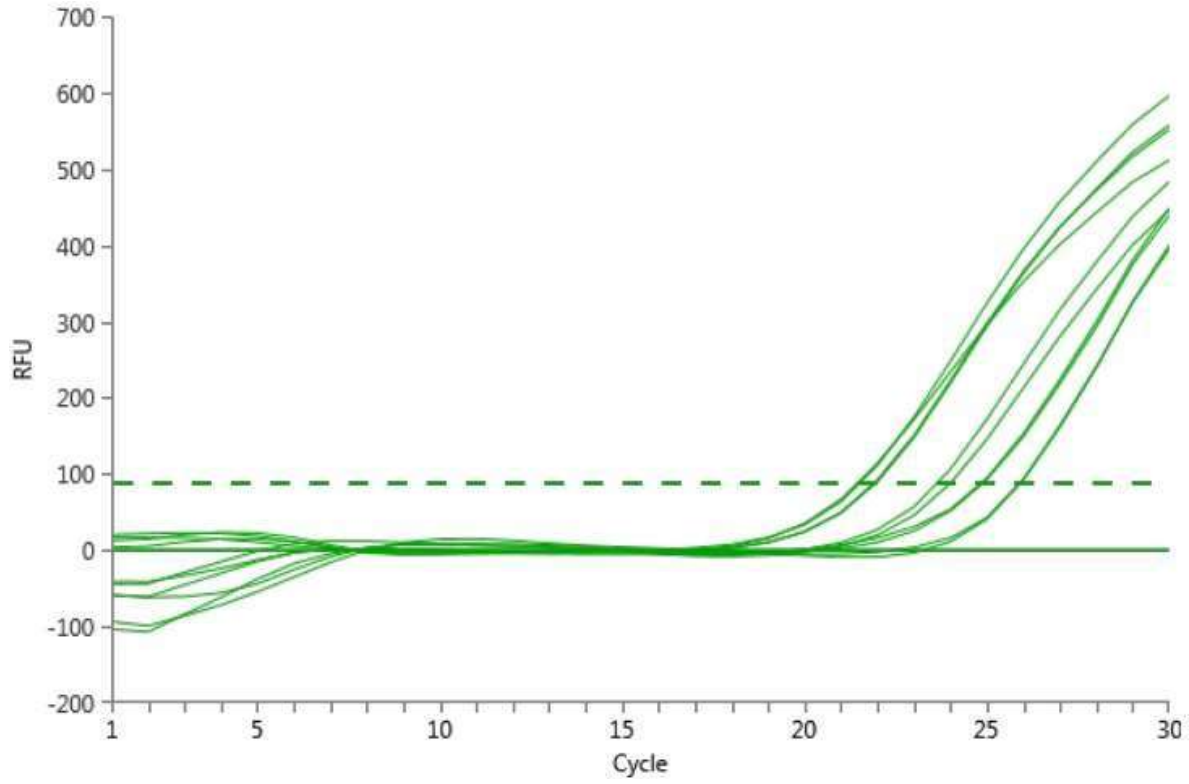


Figure 4.12 shows the amplification plot of the *Plasmodium vivax* in clinical samples

4.2.3. SENSITIVITY TESTING OF RT-PCR

The parasite count was estimated by Giemsa-stained thin blood smears examined under oil immersion (100 magnification). 410 parasites were counted against 200 white blood cells using the assumption that there are 8000 white blood cells per microliter of blood

By using the formula parasite densities of *P.vivax* was calculated

$$\text{Parasite density/ } \mu L = \frac{\text{No. of Asexual Parasites}}{\text{White blood cells counted}} \times \text{White Blood cells}$$

$$= \frac{\text{410 asexual parasites}}{\text{200 white blood cells}} \times \text{8000 white blood cells}$$

$$= \text{16,400 parasites/ } \mu L$$

The parasite density of sample was 16,400 parasites/ μL.

Whereas, the % parasitemia was estimated by the formula;

$$\text{\% Parasitemia} = \frac{\text{No. of Asexual Parasites}}{\text{Total no. White blood cells}} \times 100$$

$$= \frac{\text{410 asexual parasites}}{\text{8000 white blood cells}} \times 100$$

$$= 5.1\ \%$$

After estimation of parasite count and % parasitemia The target DNA of Plasmodium vivax was ten-fold serially diluted to contain at-least 1 parasite copy/ µL to determine the sensitivity and find the limit of detection. The limit of detection of developed Real-Time assay was 4 parasites/ul for Plasmodium vivax.

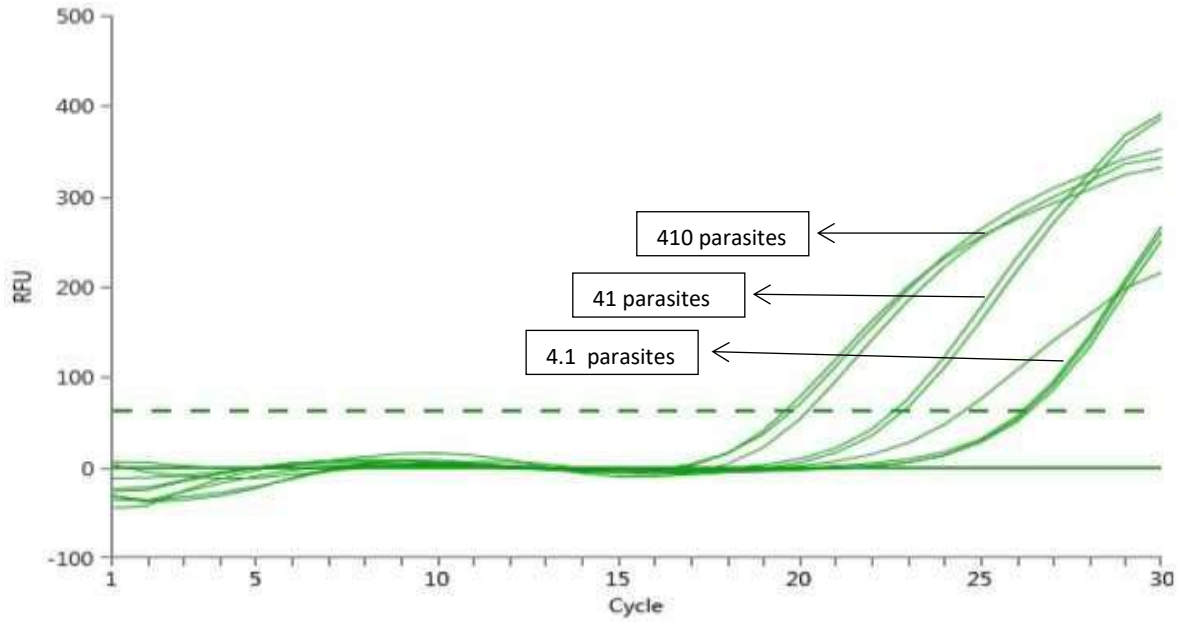


Figure4.13 shows the amplification plot of ten-fold serially diluted DNA of the target Plasmodium vivax

Ct values and log DNA concentration of 10- fold serially diluted DNA were used to generate standard curve to calculate the r2 value and efficiency of the Real-Time PCR assay.

Table 4. Standard curve was generated from the data given in the table.

Concentration DNA log	CT value
LOG(554)	19.873
LOG(55.4)	23.34
LOG(5.5)	26.26
LOG(0.5)	____

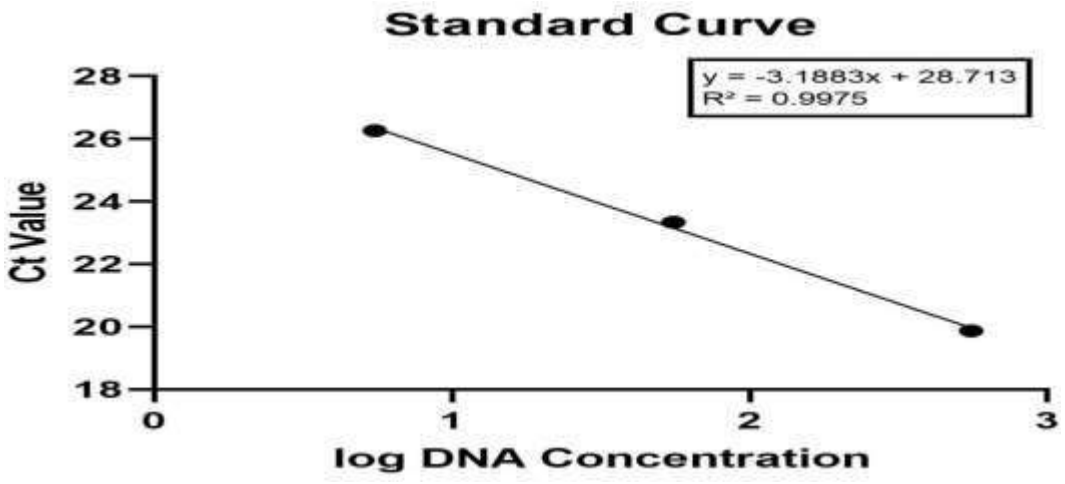


Figure 4.14 shows Standard curve analysis of 10-fold serially diluted DNAs was performed using the Graph pad prism , r2 value and assay efficiency percentage were calculated

By using the slope value, the RT- PCR efficiency was calculated using the following formula;

$$\% \text{ Efficiency} = -1+10(-1/\text{slope}) \times 100$$

$$= -1 +10\wedge(-1/3.188) \times 100$$

$$= -1+10\wedge(0.313) \times 100$$

$$= -1 +2.055 \times 100$$

$$= 1.055 \times 100$$

$$\mathbf{= 105.5\ \%}$$

The developed PCR assay efficiency was 105.5%.

4.2.4. SPECIFICITY TESTING OF RT_PCR

To find the specificity of the developed method RT- PCR was performed using human and Salmonella typhi DNA. By utilizing *Salmonella typhi* specific primers and probe an Amplification plot was produced for *Salmonella typhi* DNA as shown in figure 4.15.

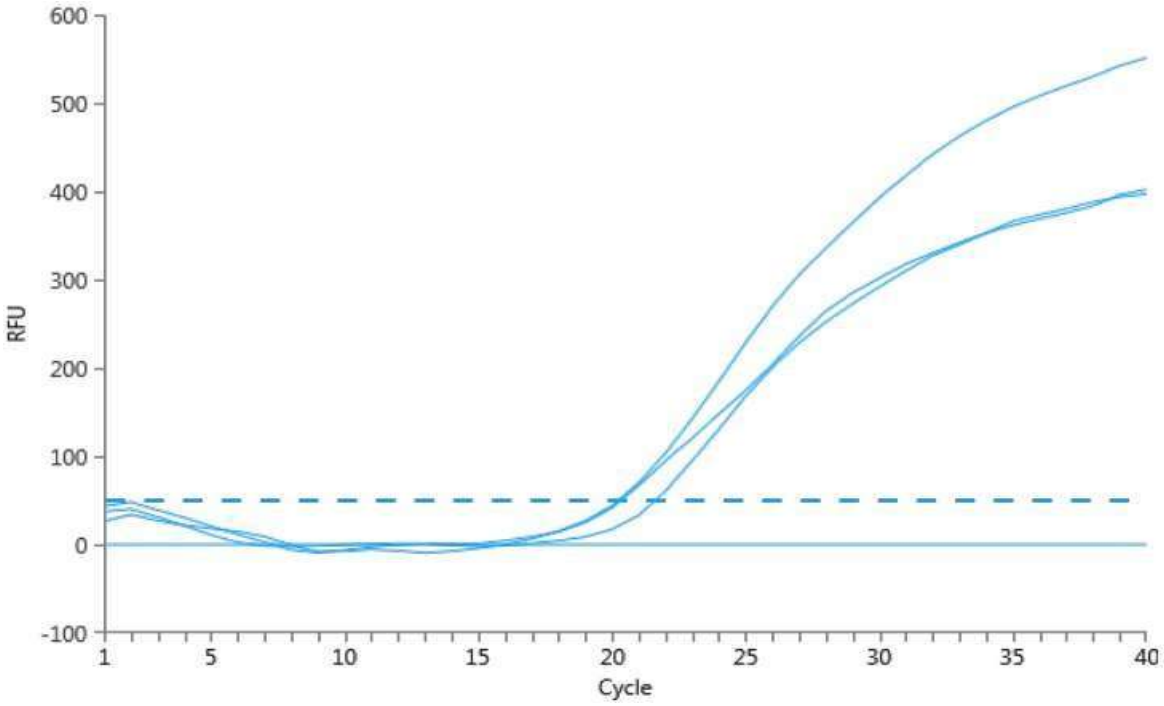


Figure 4.15 shows that amplification plot for *Salmonella typhi* DNA

Similarly, by using Human DNA and same *Salmonella typhi* DNA by using *plasmodium vivax* specific primers and probe no amplification plot was generated against the DNA of Human and *Salmonella typhi*, as can be observed in Figure 4.16. These findings demonstrated that the primer set and VIV probe was highly specific to the 18s rRNA target sequence of *Plasmodium vivax.*

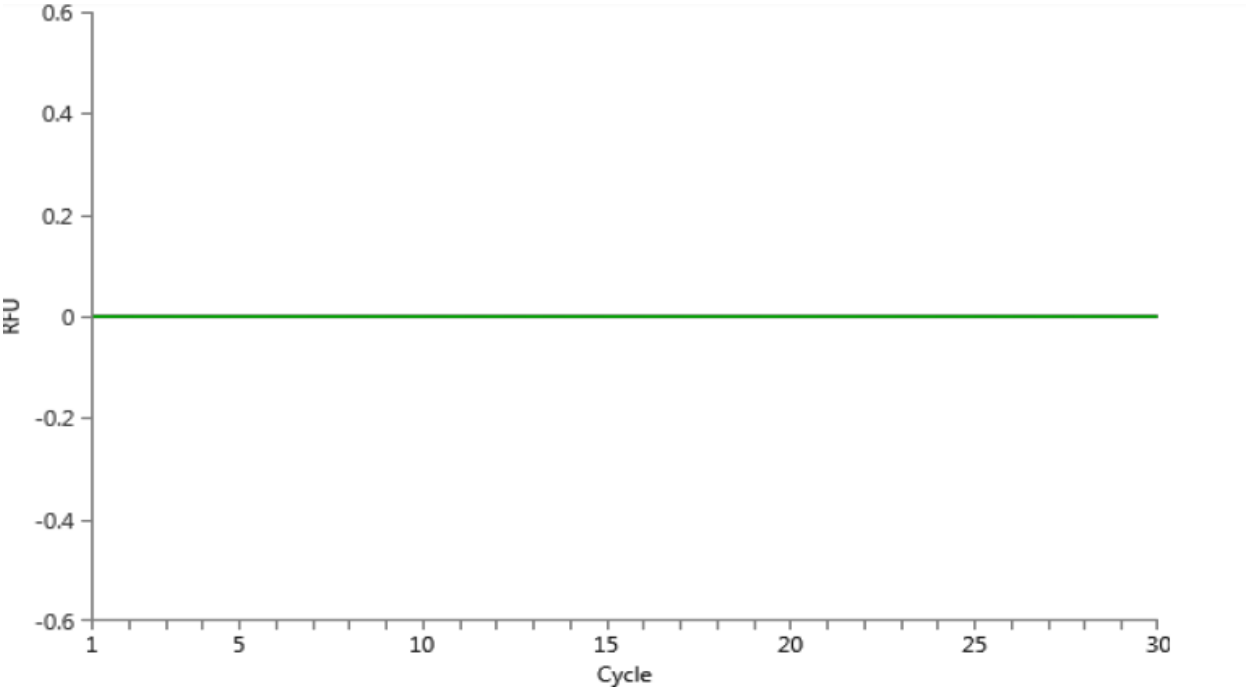


Figure 4.16 shows that no fluorescence and hence no Ct value detected for the human blood and *Salmonella typhi* DNA

5. DISCUSSION

Malaria is the 5th most common cause of mortality and is a major public health issue worldwide (*Malaria Program | Indus Hospital & Health Network*, 2022). 247,000 cases and 219,00 fatalities occurred in 2021 due to malaria. Pakistan is a country with moderate endemicity of malaria, however, 177 million people are still at risk of contracting malaria with 60% population living in endemic regions of malaria (Qureshi *et al.*, 2019). Every year Pakistan bears the burden of 3.5 million suspected and confirmed cases of malaria. About 78 % cases in Pakistan are reported of *P. vivax* and 22 % cases are of *P.falciparum* (*Malaria – Pakistan*, n.d.).

The diagnosis of malaria is still challenging due the non- specific and variable nature of symptoms that malaria produces in patients (Ghai *et al*., 2016). Microscopic examination of blood smear is the gold standard method to date for the detection of malarial parasites. When performed by a qualified microscopist, this approach is inexpensive, simple to use, enables quantification of the sample in terms of parasite count and distinguishes between the various phases of the four Plasmodium species that cause malaria in humans (Perandin *et al*., 2004b). But it is also very well known that microscopy has drawbacks, such as sensitivity falls as the density of the malaria parasite in blood decreases (Grabias *et al*., 2019). Diagnostic methods based on Real-Time PCR has overcome these drawbacks of conventional methods by providing increased sensitivity for the detection of plasmodium (Alam *et al.*, 2011). Most of these molecular diagnostic methods are based on targeting the 18s rRNA gene of Plasmodium species(Demas *et al.*, 2011).

18s rRNA gene of all plasmodium species consists of variable and conserved regions and is present in numerous copies across the plasmodium genome, making it more sensitive to amplification of target than single-copy genes. In contrast to this, the Cox1 gene is a multicopy gene which is a part

of mitochondrial genome (Ghai et al., 2016). The gene lacks highly variable regions and detection of plasmodium species utilizing this gene would require multiple primer sets and cannot serve as a target for the designing of variable specie specific probes. Therefore, in the current study 18s rRNA gene was selected for the diagnosis of malaria in clinical specimens. Forward and reverse primer targeting 18s rRNA gene of all four plasmodium species along with specie specific probes for the detection of *Plasmodium ovale, Plasmodium malariae, Plasmodium falciparum* and *Plasmodium vivax* designing and validation is described *In silico*. No primer-primer, primer-probe and probe-probe self and cross dimers existed in the FP1, RP1, Mal, Ova, Viv and Fal probes. The *Insilico* PCRs confirms the binding of FP1 and RP1 binding to the conserved region of 18s rRNA gene of all four plasmodium species with amplification products of 202,239,210 and 205 for *P.falciparum, P.malariae, P.ovale* and *P.vivax* respectively.

In the present study, only a combination of forward and reverse primer along with VIV probe is used Invitro for the detection of *plasmodium vivax* in the clinical specimens. In the 52 microscopically tested positive samples for *Plasmodium vivax* under microscopy, 31 came out negative using the current developed method. This is due to lack of both sensitivity and specificity of microscopy even though it is standard method of detection as already mentioned. Accuracy of results depend on the experience of microscopist and requires expertise. This may also be due to the presence of low parasite count in these samples, which caused insufficient extraction of parasite DNA from the samples. Poor blood film preparation can also result in artefacts that are frequently confused as parasites leading to microscopic detection of malaria (Houwen, 2002). Although, PCR-based diagnostic technologies can detect low parasite counts in comparison to microscopy, the success of diagnosis is determined by the extraction of plasmodium DNA from blood.

Method developed had a LOD of 4 parasites/ µL. The assay also showed good linearity with R2 value of 0.99 and efficiency of 105%. The current results are almost similar to that of studies like (Joste *et al*.2019) and (Murillo *et al.* 2019) which had a sensitivity of 1 parasite/ µL. (Perandin, F *et al*. 2004) developed a RT-PCR assay for detection of *P.vivax , P.falciparium, P.ovale* has shown a sensitivity 4 parasites/ul,0.7 parasites and 1.5 parasites/ µL respectively. A Real-Time PCR developed by Demas et al. *for P. falciparum-P. vivax,* also achieved similar LODs values of 1-10 parasites/ µL.

The specificity of current method was also checked against human DNA and *Salmonella Typhi* DNA as malaria shares symptoms with Typhoid fever. No amplification was observed against *Salmonella typhi* DNA indicating that Real-Time PCR is highly specific to the detection of plasmodium. These current results are similar to the results of (Rougemont et al., 2004a) (Chew *et al.,* 2012) (Lamien-Meda *et al.,* 2021b) whose developed assays did not show any amplification against Human DNA.

The real-time PCR's thermal cycling profile took a total of one hour and thirty minutes to complete. In comparison to traditional procedures based on nested and conventional PCR, real-time approaches for diagnostic purposes are quick and don't require much processing. They also don't require post-PCR gel electrophoresis for product identification(Fuehrer *et al*., 2012) (Mfuh *et al*., 2019).

The developed test was significantly more costly as compared to SYBR green methods due to use of a TaqMan probe.(Chua et al., 2015b) However, because the cost of the probe is dispersed among hundreds of PCR reactions and therefore has very low cost for a single PCR reaction. The method is still cheaper than fluorescence resonance energy transfer although the current method makes

use of TaqMan probe for the detection of *Plasmodium vivax* (Rougemont *et al*., 2004c)(Kutyavin, 2013).

6. CONCLUSION

The current study has been designed for the development of Real-Time PCR for the detection of *Plasmodium species. Invitro* the detection *of Plasmodium vivax* was analytically and clinically validated in blood specimens. Using Genomic DNA obtained from Plasmodium-infected patients, RT-PCR displayed good sensitivity for *Plasmodium vivax*. The assay also showed good linearity and is highly specific to the detection of *Plasmodium vivax* with no cross reactivity with Human DNA *and Salmonella typhi*. The Real-Time PCR assay could help with an early diagnosis in regions where there are high incidence rates of malaria. It could also help with the protection of the most at-risk group like small children and immune compromised individuals, in which *P. vivax* can be deadly in a short span of time.

7. FUTURE PROSPECTS

1.The RT- PCR developed in this study was tested on 52 microscopically positive samples in which only 21 came out positive with *Plasmodium vivax*. Therefore, the current results needs to be confirmed by using an already established Real-Time PCR.

2. Plasmid based positive controls containing the 18s rRNA fragments needs to be made for *Plasmodium malariae, Plasmodium ovale* and *Plasmodium falciparum* for validation of the results. Ova, Fal, Mal probes needs to optimized on the plasmid based positive controls.

3. After optimization, a multiplex PCR reaction needs to be developed by combining all four probes for simultaneous detection of *Plasmodium vivax, Plasmodium ovale, Plasmodium falciparum* and *Plasmodium malariae* in a single reaction.

4. Also, internal control needs to be designed for the detection of false results in the diagnostic assay.

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