DETERMINATION OF THE PREVALENCE AND DENSITY OF TRUE *PLASMODIUM FALCIPARUM* INFECTIONS AND GAMETOCYTE CARRIAGE DURING TWO MALARIA OUTBREAKS IN ZAMBEZI REGION, NAMIBIA

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Abstract

Malaria is a disease caused by parasites from the genus *Plasmodium*, which are transmitted to humans by the bite of an infected female *Anopheles* mosquito. Malaria remains as one of the leading causes of mortality globally. Namibia has successfully reduced the malaria burden by over 90% between 2004 and 2014 and aimed to eliminate the disease by 2020, however, this progress has stalled and it has experienced outbreaks. New approaches are needed to understand and address the reasons for persistent transmission and outbreaks. Accurate diagnosis is a challenge with the routinely used Rapid Diagnostic Tests (RDTs) at low transmission settings as they have been reported to give both false-positive and false-negative results, both outcomes compromise the detection and treatment of all malaria cases. Furthermore, RDTs do not give much information about epidemiological parameters that affect malaria transmission and can be used to assess and mitigate against onward transmission of the disease which includes transmission intensity and risk of transmission. Molecular tools have been reported as being able to identify true malaria infections. This includes parasite species and strains as well as being able to give a more accurate and quantitative reflection of the malaria burden and potential risk of transmission, additional information that RDTs cannot provide. This study was conducted to confirm and identify the *Plasmodium* species confirmed by RDTs; quantify parasite densities and gametocytes during the 2017 and 2021 outbreak seasons to determine the importance of these parameters in assessing transmission intensity and risk. A total of 589 malaria RDT positives, (251 from the 2017 malaria outbreak and 338 from the 2021 outbreak) from the Zambezi region with corresponding dried blood spots (DBS) were analysed using DNA extracted using the Chelex method for conventional PCR. Only 223/589 (38%) RDT positive samples

were confirmed as being true malaria positive: (71/251, 28% for 2017 and 152/338, 45% for 2021). All confirmed samples were identified as *Plasmodium falciparum* only. The QIAamp DNA kit was used to extract DNA from the 223 confirmed malaria positive samples for quantitative PCR to calculate parasite density for each malaria outbreak season (71 for 2017 and 152 for the 2021 season, a total of 223). The parasitaemia of the samples from each season was calculated and classified as follows: for 2017, low - 13%, moderate - 24%, high - 51%, hyperparasitaemia - 13%; for 2021 low - 24%, moderate - 29%, high - 40%, hyperparasitaemia - 7%. The mean parasite density was higher in 2017 (46088 p/µL) as compared to 2021 (35211 p/µL) (Mann-Whitney, p=0.008). Gametocyte carriage was determined by RTqPCR to detect and quantify the presence of *Pfs25* transcripts for female gametocytes. The study showed that as parasitaemia increased, there was a higher chance of detecting gametocytes. Gametocyte detection was found in 59/71 (83%) for 2017 and 98/152 (64%) for 2021 using a Ct value of ≤35 as a cut-off point. There was a significant difference in gametocyte mean for 2017 (116 g/µL) and 2021 (892 g/µL) (Mann-Whitney, p=0.003). There was a moderate correlation between parasitaemia and gametocyte carriage during the 2017 outbreak (*rho* = 0.6) and a strong correlation for the 2021 outbreak (*rho* = 1) using Spearman's Correlation. The Median test showed that during the 2021 outbreak there was no significant difference (p=0.910) between gametocyte carriage in low parasitaemia infections and moderate parasitaemia infections. This showed that low density infections are actually a risk factor for onward transmission of malaria. This study confirmed that *P. falciparum* is the dominant parasite causing malaria in the Zambezi region during the 2017 and 2021 outbreaks. It also showed that the use of RDTs for Reactive Case Detection (RACD) does not give an accurate picture of true malaria cases during outbreaks, only 38% of cases were confirmed by

PCR. Both outbreaks showed moderate parasite density on average although both had high parasitaemia and hyper parasitaemia cases, signals indicative of an outbreak. Parasitaemia also correlated with gametocyte carriage showing that the parasite density was linked with higher transmission risk through gametocyte carriage. Thus, parasite density and gametocyte carriage as determined through the use of molecular tools can be used to assess the risk of transmission. However, further studies should be conducted in dry seasons to have a better understanding of residual transmission, parasite reservoir, and malaria epidemiology as a comparison to the outbreak seasons. The use of additional molecular analysis such as the Multiplicity of Infection (MOI), heterozygosity index and parasite transmission networks can also enrich the data collected during molecular surveillance and its usefulness in heterogeneous malaria transmission settings. It is recommended that molecular surveillance be used during passive surveillance at clinics; used during reactive case detection and Mass Screen and Treat and when targeting interventions to high-risk groups to understand the drivers of sustained transmission. This will contribute to the tailoring of cost-effective interventions and not a one-size fits all blanket intervention.

Keywords: Quantification, *Plasmodium*, Rapid Diagnostic Tests, Dried Blood Spots, Surveillance, Parasitaemia, Gametocytes

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List of Abbreviations and Acronyms

µL: Microlitre

ACD: Active Case Detection

ACT: Artemisinin-based Combination Therapy

AL: Artemether-Lumefantrine

bp: base pairs

COVID-19: Coronavirus Disease 2019

Ct: Cycle Threshold

DBS: Dried Blood Spot

DNA: Deoxyribonucleic acid

DNTPS: Deoxynucleotide Triphosphates

E8: Elimination 8

g/µL: gametocytes per microlitre

G6PD: Glucos-6-Phospahte Dehydrogenase

HRP2: Histidine Rich Protein 2

IPTi: Intermittent Preventative Treatment in infants

IPTp: Intermittent Preventative Treatment in Pregnant Women

IRS: Indoor Residual Spray

LAMP: Loop-Mediated Isothermal Amplification

LLINs: Long Lasting Insecticide-Treated Nets

LOD: Limit of Detection

LSM: Larval Source Management

MDA: Mass Drug Administration

MoHSS: Ministry of Health and Social Services

MOI: Multiplicity of Infection

MSAT: Mass Screening and Treatment

NASBA: Nucleic Acid Sequence Based Amplification

NEC: Negative extraction control

nPCR: nested Polymerase Chain Reaction

NTC: Negative template control

NVDCP: National Vector-borne Disease Control Program

p/µL: parasites per microlitre

PCR: Polymerase Chain Reaction

PEC: Positive extraction control

pfhrp2/3: *Plasmodium falciparum* histidine rich protein 2/3

pLDH: *Plasmodium* Lactate Dehydrogenase

PTC: Positive template control

QA: Quality Assurance

qPCR: Quantitative Polymerase Chain Reaction

RACD: Reactive Case Detection

RDTs: Rapid Diagnostic Tests

rfMDA: reactive focal mass drug administration

RNA: Ribonucleic Acid

RTqPCR: Reverse Transcriptase Quantitative Polymerase Chain Reaction

SADC: Southern African Development Community

SARS: Severe Acute Respiratory Syndrome

SMC: Seasonal Malaria Chemoprevention

UV: Ultra Violet Light

varATS: var gene Acidic Terminal Sequence

WHO: World Health Organization

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Great things are not done by one person. They are done by **a team of people…**

A supervisor, in my opinion should challenge you for greater, inspire you and instil intentional purpose in your research endeavour, and you Prof Davis R. Mumbengegwi have done this. Thank you.

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Dedication

I humbly dedicate this to you, the reader. May every page you read allow you to see and experience the thought-processes, time, effort, commitment and dedication all mixed together to bring about this 100+ page document.

I surely enjoyed every moment on this journey (the highs and lows of progress and getting good results, having to try again and again, the trouble shooting, the back to square one and the Eureka moments). Seeing this document come to life and being read by you is indeed a source of pride and there is fulfilment in starting **AND** finishing something.

Indeed, Rome was not built in one day…

Declaration

I, Lydia Eloff declare that this study is a true reflection of my own research, and that this work, or part thereof has not been submitted for a degree in any other institution of higher education.

Name of Student……. Signature…… Date…….

Chapter 1. Introduction

1.1 Background of the Study

1.1.1 Malaria and its Global Health Burden

Plasmodium parasites are single-cell protozoa and are the causative agents of malaria in humans and other species which include, primates, rodents and even birds (Sato, 2021). Malaria is communicable and life-threatening, yet it is a preventable disease (Yin, Yan & Li, 2022). The disease is transmitted by a female mosquito vector that belongs to the genus *Anopheles* (Adedeji et al., 2020; Yin, Yan & Li, 2022). These vectors thrive in warm temperatures with high humidity and high rainfall, typically in tropical and subtropical regions which allow the *Anopheles* mosquito to survive and multiply as well as allow the *Plasmodium* parasite to complete its growth cycle within the mosquitoes (Kipruto et al., 2017). There are 5 species within this genus known to cause malaria in humans, namely: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae* and *Plasmodium knowlesi* (Kwenti et al., 2017; Tizifa et al., 2018; Idárraga et al., 2021; Oyegoke, Maharaj, et al., 2022). The most prevalent and fatal species worldwide is *P. falciparum*, accounting for 99.7% of estimated malaria cases in the World Health Organization (WHO) African region (Idárraga et al., 2021).

Globally, an estimated 241 million malaria cases were reported in 2020 and the recorded malaria deaths increased by 12% compared with 2019. A total of 627 000 deaths were recorded and about 47 000 deaths were due to service disruptions in the provision of malaria prevention, diagnosis and treatment during the COVID-19 pandemic (World Health Organization, 2021). Malaria transmission occurs in five World Health Organization (WHO) regions namely: the WHO African Region, the WHO South-East Asia Region, the WHO Eastern Mediterranean Region, the WHO

Western Pacific Region and the WHO Region of the Americas (World Health Organization, 2020). The WHO African Region continues to carry a disproportionately high share of the global malaria burden. In 2020 the Region had an estimated 228 million cases which accounted for 95% of all malaria cases and 96% of deaths. Children under 5 years of age accounted for about 80% of all malaria deaths in the Region (World Health Organization, 2021). The WHO global malaria strategy urges all malaria-endemic countries to accelerate progress towards the goal of malaria elimination defined as "the interruption of local transmission of a specified malaria parasite species in a defined geographical area as a result of deliberate activities" (World Health Organization, 2021). In settings approaching elimination such as Namibia, interventions will be most effective at reducing transmission if they are tailored to detect and treat the residual foci of malaria transmission (Gosling et al., 2020).

1.1.2 Malaria in Namibia

Namibia, a Southern African country is classified as a low malaria transmission setting (< 100 Annual Parasite Incidence recorded in the last 5 years) with transmission risk zones varying geographically from 'moderate' to 'risk-free' (Nghipumbwa et al., 2018). The country is targeting malaria elimination by 2023 (Raman et al., 2021). Malaria transmission is known to be clustered in northern Namibia and is seasonal with peaks between December and April which are highly dependent on rainfall patterns (Jacobson et al., 2019). Low transmission settings like northern Namibia which borders other malaria-endemic countries such as Angola, Zambia and Botswana (Smith et al., 2021) present unique challenges for surveillance given the small number of infected individuals and increased heterogeneity of transmission (Jacobson et al., 2019). The figure below shows northern Namibia bordering Angola, Zambia and

Botswana with an oval red circle which are other malaria-endemic countries where cross-border importation of malaria occurs.

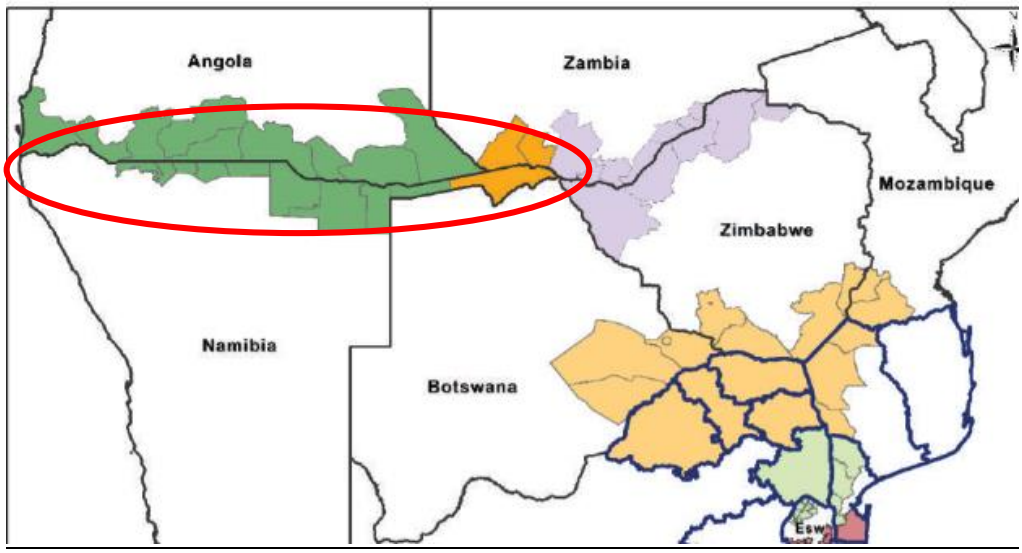


Figure 1: Map illustrating Northern Namibia bordering other malaria-endemic countries

Source: (Elimination 8, 2021)

Malaria surveillance in low transmission settings is important to understand the causes of the outbreaks. The surveillance system consists of the tools, procedures, people and structures that generate evidence-based information on malaria cases and deaths, which can be used for planning, monitoring and evaluating malaria control programs (World Health Organization, 2012). An effective malaria surveillance system enables program managers to identify the areas or population groups most affected by malaria, identify trends in cases and deaths that require additional intervention and assess the impact of the control measures used. With such information, programs can direct resources to the populations most in need and respond to and understand outbreaks from the generated evidence-based information (World Health Organization, 2012).

Namibia has successfully reduced the burden of malaria through the large-scale deployment of the following: long-lasting insecticide-treated nets (LLINs), indoor residual spraying (IRS) (Mumbengegwi et al., 2018), rapid diagnostic tests (RDTs) and artemisinin-based combination therapy (ACT) when malaria positive, (McCreesh et al., 2018; Jacobson et al., 2019; Smith et al., 2021).

Namibia has experienced fluctuating malaria burden over the past years, due to seasonal outbreaks. Although the country has made significant progress over the last 10 years (Smith et al., 2021), the ongoing threat of malaria importation from across the country's borders is a key barrier to achieving elimination. Human migration from endemic to lower transmission areas places the destination countries at risk for malaria outbreaks or resurgence. (Gueye et al., 2014). Namibia is a member of the SADC Malaria Elimination eight (E8) initiative where eight countries in Southern Africa [Figure 2] have pledged to work together to employ cross-border interventions to control and eliminate malaria in the countries by 2030 (Elimination 8, 2019). The E8 consists of Namibia, Botswana, Eswatini (previously known as Swaziland) and South Africa, which are the four frontline countries, and Angola, Zambia, Zimbabwe and Mozambique, which are the four second line countries (Elimination 8, 2021; Raman et al., 2021). Malaria elimination is defined as: "the interruption of local mosquito-borne malaria transmission; reduction to zero of the incidence of infection caused by human malaria parasites in a defined geographical area as a result of deliberate efforts; and continued measures to prevent reestablishment of transmission" (Autino et al., 2012; Gueye et al., 2014). Below is a map showing the E8 frontline and second line countries.

Figure 2: Map showing SADC Malaria Elimination eight countries in Southern Africa including the frontline and second line countries.

Source: (Elimination 8, 2019)

The World Health Organization (WHO) developed a malaria elimination continuum to assist control programs in malaria-endemic countries to determine their malaria status, using malaria incidence [Figure 3]. Countries with a malaria blood-slide positivity rate among fever cases >5 cases/1000 population at risk are considered to be in the malaria control stage; <5 and >1 case/1000 are in the pre-elimination stage; and at rates <1 case/1000 are categorised as being in the elimination stage (Moonasar et al., 2013). The path to elimination is a continuum from control of the disease including the prevention of death to the documentation of zero infections and no local

transmission (Wirth et al., 2018). Due to the outbreaks experienced by Namibia, the country was not included in the E-2025 initiative by the WHO, a list of countries identified as most likely to eliminate malaria by 2025. Below is an image showing the malaria elimination continuum.

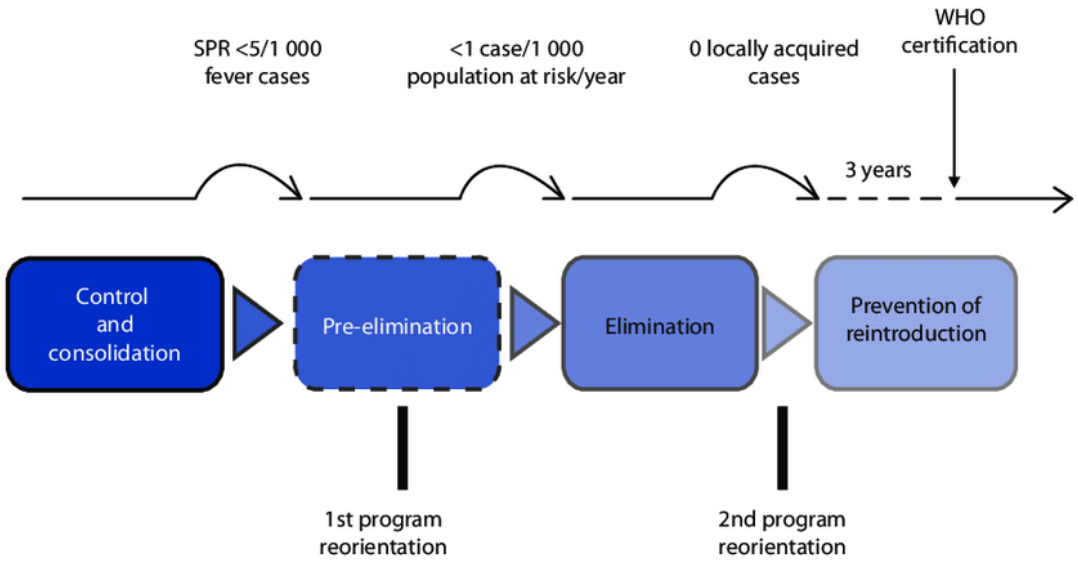


Figure 3: World Health Organization malaria elimination continuum.

Source: (World Health Organization, 2008)

Malaria in Namibia is heterogeneous with the majority of the cases coming from Kavango and Zambezi region. Both Kavango and Zambezi regions have contributed to over 80% of the reported malaria cases in the country. Figure 4 below shows the Namibian map with higher incidence reported in the Kavango region in 2017-2018 and an increase in incidence in the Zambezi region in 2020-2021.

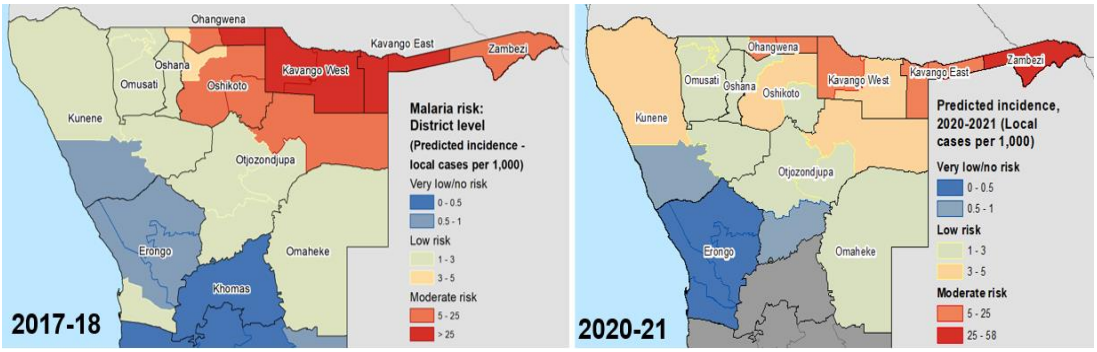
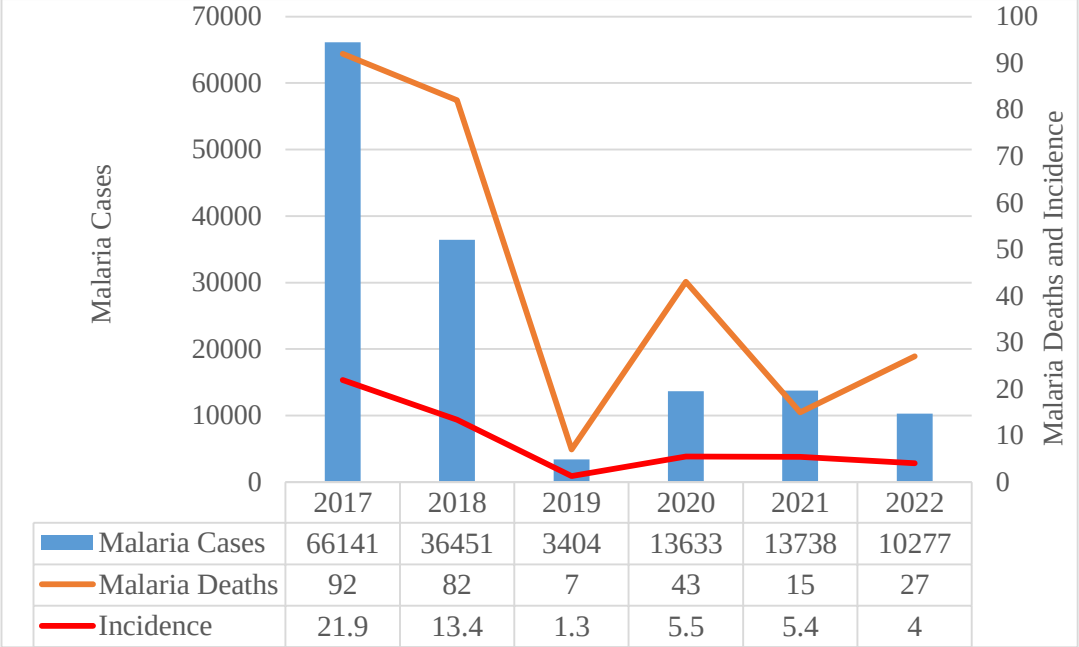


Figure 4: Namibian map with the case level incidences for the years 2017-18 and 2020-21

Source: (NVDCP, 2022) Ministry of Health and Social Services Malaria Program Review, 2022

Namibia has experienced fluctuating numbers of malaria cases showing the vulnerability of the country to outbreaks, possibly due to importation and residual transmission of malaria. From the years 2017 to 2022, 66141 cases were reported in 2017, 36451 in 2018, 3404 in 2019, 13633 in 2020,13738 in 2021 and 10277 in 2022 (NVDCP, 2022). The different case number for 2019 was due to a severe draught. Figure 5 below shows the summarised malaria cases, deaths and incidence in Namibia.



	2017	2018	2019	2020	2021	2022
Malaria Cases	66141	36451	3404	13633	13738	10277
Malaria Deaths	92	82	7	43	15	27
Incidence	21.9	13.4	1.3	5.5	5.4	4

Figure 5: The Malaria cases, deaths and incidence in Namibia from the years 2017-2022.

Source: (NVDCP, 2022) National Malaria Strategic Plan 2017-2021 review meeting

Zambezi region, the site of this study, is located in the northern-eastern part of Namibia and shares borders with Angola and Zambia, which are malaria-endemic countries. Cross-border malaria transmission through population movement from these countries contributes to the challenges Namibia faces in eliminating malaria (Gueye et al., 2014). It is the wettest region in Namibia with the wet season between October to May (McCreesh et al., 2018; Mumbengegwi et al., 2018). High transmission begins in the month of November, then peaks between January and April then trails of in May. The parasite incidence in Zambezi region from the years 2004 and 2015 was 600 and 17.1 cases per 1000 respectively, over a period of 10 years (McCreesh et al., 2018). This decline in incidence is attributable to the previously mentioned scale-up use of interventions: LLINs, IRS, RDTs, and treatment with ACTs for those confirmed positive with malaria (McCreesh et al., 2018; Jacobson et al.,

2019). Below is a graph showing the recorded number of cases specifically in the Zambezi region (Figure 6).

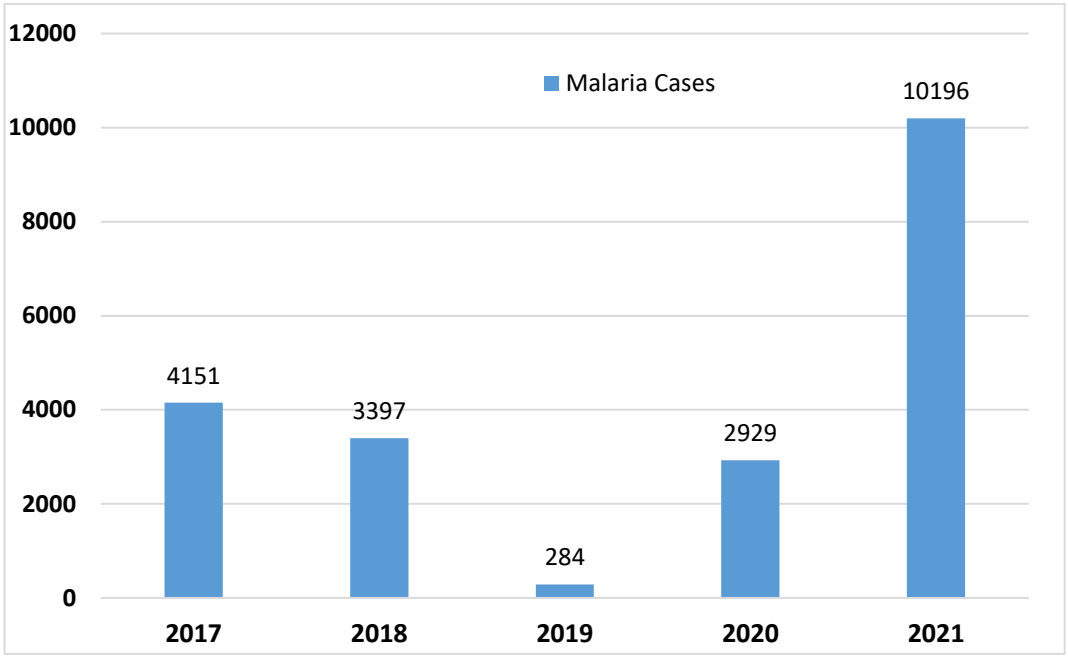


Figure 6: Malaria cases in Zambezi region 2017-2021.

Source: (MoHSS, 2022) Ministry of Health and Social Services Malaria Program Review, 2022

1.1.3 Challenges Namibia Faces in Achieving Malaria Elimination

Despite interventions such as LLINs and IRS, the main vector control methods currently implemented, outdoor and evening or early morning malaria transmission still occurs in many malaria-endemic regions and is considered residual malaria transmission (Mundagowa & Chimberengwa, 2020; Nalinya, Musoke & Deane, 2022). Residual malaria transmission is therefore defined as all forms of malaria transmission that persist even after full coverage with effective LLIN and/or IRS interventions have been achieved (Killeen, 2014). Residual transmission measures all forms of transmission that are beyond the reach of standard insecticidal nets and indoor residual spraying of insecticides when used optimally. The epidemiological

importance of the time of day mosquitoes bite and how much this contributes to residual transmission is unclear (Sherrard-Smith et al., 2019).

Namibia had aimed towards malaria elimination by 2020, but has experienced several malaria outbreaks in the north since 2016 (Smith et al., 2021), leading to the adjustment of the elimination date to 2023 (Raman et al., 2021). Challenges that compromise this goal of eliminating malaria in Namibia include diagnostic tools (microscopy and RDTs) that are not sensitive enough to detect all cases, especially at low parasitaemia (Dahal et al., 2021). Reports show that in low-transmission settings, asymptomatic infections are common and most of these infections are sub-microscopic. Asymptomatic malaria carriers do not seek treatment and they may serve as reservoirs of infection, jeopardizing elimination efforts (Tambo et al., 2018; Thompson et al., 2020). The challenges asymptomatic malaria infections pose to malaria elimination efforts are also exacerbated by the diagnostic limitations in detecting the infections at low parasite densities (Zemene et al., 2018). Asymptomatic malaria infections that remain undiagnosed and are not treated are largely neglected by most of the currently implemented malaria interventions and control programs and they contribute to onward transmission (Mobegi et al., 2021).

RDTs can also result in false-positives due to antigen persistence and when Reactive Case Detection (RACD) leads to repeat visits around index case neighbourhoods that are near each other (Sturrock et al., 2013). Another challenge is due to importation, pockets of transmission remain primarily in northern border areas where malaria receptivity remains high and vulnerability is greater due to continuous population movement from neighbouring endemic countries (Gueye et al., 2014). There is a higher risk of infection in individuals known as high-risk populations engaged in agricultural occupations and cattle herding, as reported in other low transmission

settings, outdoor occupations may increase exposure to mosquito bites facilitating onward transmission (McCreesh et al., 2018).

To reduce or interrupt malaria transmission, it is important to find and treat all infections which can perpetuate transmission. As such, using RACD for surveillance to detect and treat secondary cases is widely practiced in low-endemic settings (Hsiang, Ntshalintshali, et al., 2020). RACD [Figure 7], is triggered by passively detected cases at health facilities and involves screening households or individuals within a specified area (within a pre-determined radius), around a local or imported case, with the goal of preventing further malaria transmission by identifying additional infections, both symptomatic or asymptomatic (Perera, Caldera & Wickremasinghe, 2020). The rationale for RACD is that asymptomatic or minimally symptomatic malaria are geographically clustered in space and time around index households (Hsiang, Ntshalintshali, et al., 2020). RACD and Mass Screening and Treat (MSAT) approaches are used to identify and treat malaria cases (World Health Organization, 2022). Below is a figure showing the method of RACD.

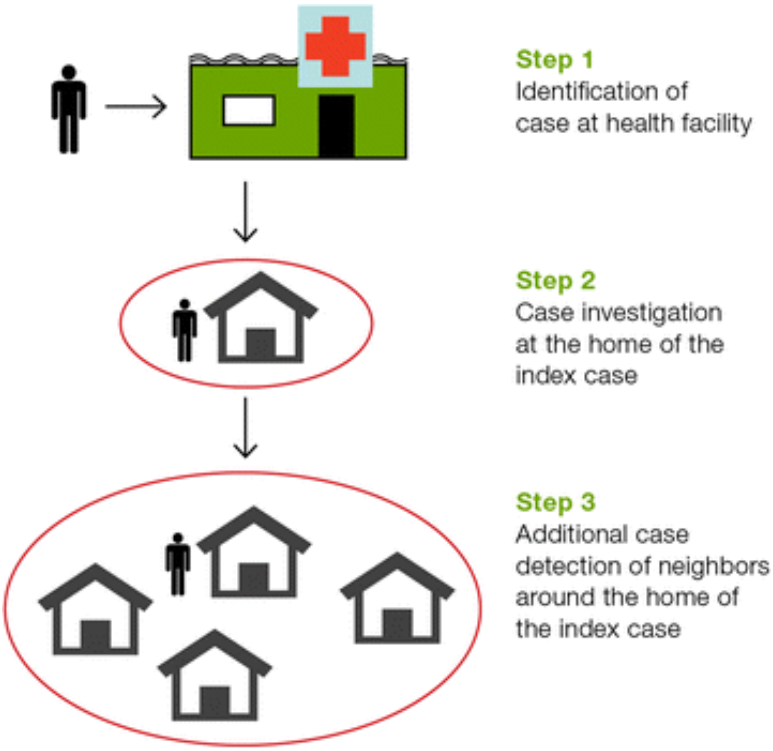


Figure 7: Illustration of steps in Reactive Case Detection (RACD).

Source (Cotter et al., 2017)

The effectiveness of these strategies are however limited in populations where treatment-seeking is low; where infections are asymptomatic; or the population is mobile, and residents are frequently absent such as in agricultural seasons (Smith et al., 2021). Also reliance on conventional RDTs limits RACD's effectiveness due to sensitivity (Hsiang, Ntshalintshali, et al., 2020). Smith et al., (2017) showed that secondary infections have been shown to cluster around index cases and the reduced sensitivity of RDTs limit the effectiveness of RACD to identify and treat infections (Smith, Auala, Tambo, et al., 2017). In Namibia and other low endemic settings in southern Africa, RACD using RDTs only identifies up to one-third of infections detected by more sensitive molecular methods (Smith, Auala, Tambo, et al., 2017). An intervention that circumvents the limitation of RDT sensitivity during RACD is reactive focal mass drug administration (rfMDA), defined as mass drug administration

targeting household members and neighbours of recent passively detected cases. Since RDTs have limited sensitivity, the people in close proximity to the index cases are all treated since they are known to be at risk of malaria (Hsiang, Ntuku, et al., 2020)

Surveillance is an important component of an effective system to support malaria elimination. However poor surveillance data can prevent countries from monitoring progress towards elimination and targeting interventions (Lourenço et al., 2019). A better understanding of the prevalence and characteristics of all infections based on strong surveillance systems can lead to improved timely and targeted strategies and interventions for malaria elimination (McCreesh et al., 2018). For currently used interventions to continue succeeding, the human reservoir of malaria needs to be identified and patterns in its infectiousness need to be understood to optimize control interventions (Ouédraogo et al., 2016). Using inaccurate and incomplete information obtained from the diagnostic tools that miss low parasite densities does not allow malaria control programs to fully understand the true infection burden and to accurately assess transmission risk (Okell et al., 2012). Therefore, understanding the contributing reasons to outbreak occurrence in low transmission settings enables to provide early case management, identify factors that maintain the disease, design and improve more effective prevention and control measures to facilitate malaria elimination by 2030 (Tesfahunegn, Berhe & Gebregziabher, 2019).

1.1.4 Malaria and COVID-19

The COVID-19 pandemic in Namibia is part of the worldwide pandemic of coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). On 12 January 2020, the World Health Organization (WHO) confirmed that a novel coronavirus was the cause of a respiratory illness in a cluster of people in Wuhan City, Hubei Province, China, which

was reported to the WHO in December 2019 (Adhikari et al., 2020). The COVID-19 epidemic in Africa threatened malaria control with shifted attention mostly to COVID19 with malaria cases not receiving attention and attendant risk of missed or delayed malaria diagnosis and treatment (Chung et al., 2022). Already-fragile health-system capacity risked being overwhelmed, meaning access to primary care for routine case management and the availability of hospitalisation for severe malaria decreased sharply. The delivery of malaria control across the continent relies on workforces distributed across multiple sectors, which include front-line health workers, health-system administrators, logistic and field personnel carrying out and orchestrating community-based intervention delivery (Weiss et al., 2021).

The COVID – 19 pandemic resulted in a disruption of this system as a result of widespread absenteeism, movement restriction and/or diversion of workforce towards the pandemic. This in turn led to the neglect of malaria control efforts. The supply chains that allow malaria control commodities such as antimalarial drugs, bed nets, rapid diagnostic test kits, and insecticide to be manufactured, procured, and delivered internationally and within Africa were impacted by movement restrictions and lockdowns (Ajayi, Ajumobi & Falade, 2020; Weiss et al., 2021; Hakizimana et al., 2022). Namibia's main primary vector control, IRS coverage declined to below the WHO recommended levels >85% which resulted in possible outbreaks (NVDCP, 2021).

Despite restrictive measures for COVID-19 control, there are immense opportunities as well for malaria control. Leveraging existing system and resources is very important at this time and will support both the control of malaria and COVID-19 especially in rural and hard-to-reach areas. These include integrated testing, surveillance, net distribution, and malaria prevention and health promotion messaging, contact tracing

with immediate follow up during case investigation and management, leveraging focused systemic efforts for COVID-19 (Ajayi, Ajumobi & Falade, 2020). Strong health information systems enable people to test, diagnose, quarantine, and manage cases; exchange information, analyse data, and rapidly share results of those analyses, which are all essential steps to create an effective pandemic response that can be applied to the malaria situation as well. Strong health information systems are the combined power of national diagnostic networks, laboratory systems, supply chains, and surveillance mechanisms working together. Existing infrastructure for COVID-19 supplies distribution should be leveraged for malaria commodities distribution especially in rural and hard-to-reach areas and the proactive surveillance for COVID-19 should be used to strengthen malaria and other diseases surveillance in order to address global health security (Ajayi, Ajumobi & Falade, 2020). In Namibia, major surveillance challenges include reporting delays and inconsistent case investigation practices. To achieve zero transmission, case origins should be determined through comprehensive investigations followed by immediate reactive case detection to find other infections using molecular diagnostics, including asymptomatic infections that would not otherwise be identified through passive case detection at health facilities (Gueye et al., 2014).

1.1.5 Symptomatic and Asymptomatic Malaria

Malaria shares similar symptoms with other febrile diseases such as dengue fever, typhoid fever, common cold, respiratory tract infection, dyspepsia, and pneumonia. Due to this, parasitological tests, in the form of a microscopic test and a rapid diagnostic test (RDT) are necessary to confirm whether potential patients suffer from malaria or not, as incorrect misinterpretation of the disease may lead to misdiagnosis and inappropriate presumptive treatment (Bria, Yeh & Beding, 2021). Malaria

symptoms include: fever, headache, weakness, myalgia, chills, dizziness, abdominal pain, diarrhoea, nausea, vomiting, anorexia, and pruritus (Tangpukdee et al., 2009). Most of these symptoms overlap considerably with other common, as well as potentially life-threatening diseases mentioned previously. Due to this reason, WHO has recommended that all cases of suspected malaria should undergo a parasitological confirmed test (Iwuafor et al., 2019). Symptomatic individuals usually have higher treatment seeking behaviour as compared to asymptomatic cases and on diagnosis, are treated with both a gametocytocidal and a blood schizonticide agent, such as Primaquine and artemisinin-based combination therapy (Kosasih et al., 2021).

Asymptomatic malaria is defined as having parasites in peripheral circulation in the absence of symptoms that are associated with malaria infections such as fever and chills, in the absence of any antimalarial treatment (Acharya et al., 2017; Tamiru, Tolossa & Regasa, 2022). When transmission reaches low levels, sub-microscopic carriers are estimated to be the source of about 20–50% of all human-to-mosquito transmissions (Okell et al., 2012). In endemic areas, four to five asymptomatic carriers are found for every symptomatic malaria case, moreover, a small percentage of infected persons is sufficient to re-start malaria transmission in regions where there is seasonal and low transmission of malaria (Abebaw et al., 2022). Asymptomatic malaria infections likely serve as an important parasite reservoir by maintaining transmission, as parasites from asymptomatic carriers are more infectious to the *Anopheles* mosquito and a major source of gametocytes for local mosquito vectors, contributing to the persistence of malaria transmission (Abebaw et al., 2022). Since gametocytes facilitate onward transmission, studies focusing on gametocytes by looking at female specific *Pfs25* transcripts for example can assess transmission risk (Jong et al., 2020).

Current interventions mostly target passive cases detected (those who present themselves at clinics or hospitals) or vulnerable groups such as pregnant women and children. Although current interventions have been shown to drastically reduce malaria prevalence, they remain insufficient in meeting the goal of reducing the prevalence of malaria to elimination levels, as they do not target all cases, especially asymptomatic malaria or low parasitaemia (Ndong et al., 2019). When mosquito population numbers are low, asymptomatic infections in humans become a refuge for the parasite population resulting in these infected asymptomatic individuals becoming the source of infection at the beginning of the malaria season (Stresman et al., 2010). Understanding the contributions of both symptomatic and asymptomatic infections to onward transmission of malaria parasites to mosquitoes can provide guidance towards interventions to reduce and eliminate transmission. These contributions depend on parasite density, infection duration, care-seeking behaviour, and importantly - gametocyte production (Andolina et al., 2021). To meet the elimination target, identifying parasite density and gametocyte carriage in all cases during surveillance can provide evidence-based information for where to target tailor made strategies. These strategies that interrupt transmission should include early, prompt identification and treatment of all infections (Naing et al., 2022).

1.1.6 Laboratory Diagnosis of Malaria

The non-specific nature of the clinical signs and symptoms for malaria may result in misdiagnosis of the disease resulting either in over-treatment of malaria or non-treatment of other diseases in malaria-endemic areas (Tangpukdee et al., 2009). The quality of malaria diagnosis is important in all settings, as misdiagnosis can result in significant morbidity and mortality (Berzosa et al., 2018). In the laboratory, malaria is diagnosed using different techniques such as conventional microscopic diagnosis by

staining thin and thick peripheral blood smears which allows the microscopist to detect, identify and quantify the parasitaemia (Dhorda et al., 2020). Parasite detection and quantification of *Plasmodium* infections, especially *Plasmodium falciparum* (as it accounts for the highest burden in Sub-Saharan Africa) are of prime importance to ensure patients positive with malaria are treated with the most appropriate antimalarial treatment based on the type and severity of the infection (Dhorda et al., 2020). The WHO recommends the use of microscopy and RDTs for diagnosis of malaria in clinical settings (Shankar et al., 2021), and molecular tools such as PCR and LAMP as quality control for RDTs in low transmission settings. As RDTs often miss sub-patent infections which make up the majority of cases in low transmission settings (Tambo et al., 2018).

Diagnosing asymptomatic and low density infections are not as straightforward due to the lack of clinical manifestations and often sub patent level of parasites. Molecular diagnostic methods, such as polymerase chain reaction (PCR) have proven to be one of the most specific and sensitive diagnostic methods, particularly for malaria cases with low density or mixed infection (Tangpukdee et al., 2009). Other laboratory diagnostic tools include: loop-mediated isothermal amplification (LAMP), flow cytometry assay techniques, and serology. These tools have permitted extensive characterization of the malaria parasite (Tangpukdee et al., 2009). Accurate diagnosis and surveillance of malaria is important for providing realistic estimates of the malaria burden and preventing misinformed interventions due to incomplete and inaccurate information (Jiram et al., 2019).

1.1.7 Challenges with WHO Recommended Diagnostic Tools

Microscopy and rapid diagnostic tests (RDTs) are the main techniques used to diagnose clinical malaria for parasitological confirmation as recommended by the

WHO (Iwuafor et al., 2019). Microscopy allows for parasite detection, identification and quantification and is useful during follow-up of parasitaemia and assessments of the therapeutic efficacy of antimalarial drugs (Horning et al., 2021). While microscopy is still considered the gold standard, the tool has a detection limit of about 100-200 parasites/µL (Das et al., 2018; Picot, Cucherat & Bienvenu, 2020). Misdiagnosis by a microscopist deeming the result as negative, when the patient is actually malaria positive can result in significant morbidity and mortality (Berzosa et al., 2018). Also misdiagnosis by a microscopist deeming the result as positive due to artefacts in low-quality blood smears can lead to false-positive readings by untrained technicians leading to overtreatment with artemisinin-based combination therapy (ACTs) which may lead to the progression of resistance and a waste of resources (Tedla, 2019; Fitri et al., 2022).

RDTs are immuno-chromatographic lateral flow devices that are simple to use, allow for rapid, inexpensive detection, easier to carry and require less experienced/qualified personnel and they gives results from whole blood within 20 minutes (Bamou et al., 2021). They do not require the need for electricity and special equipment, as compared to microscopy and can be used in rural/remote/limited resource settings (Fitri et al., 2022). However, the sensitivity of RDTs is dependent on the level of parasitaemia in the patient, that is, they have poor performance in terms of sensitivity for low density parasite infections with detection limits at approximately 100–200 p/µL which limits their utility in elimination settings (Das et al., 2018; Nundu et al., 2022). RDTs can also result in false-positives due to persistent antigenicity from previous infections, other infectious agents or immunological factors overestimating prevalence. Also deletions in the pfhrp2 gene can lead to false-negatives (Apinjoh et al., 2021; Bamou et al., 2021; Manjurano et al., 2021). RDTs only provide qualitative information and

do not quantify parasitaemia or differentiate parasite stages which are important in the determination of malaria severity, prognosis and transmission risk (Idárraga et al., 2021; Agbana et al., 2022; Fitri et al., 2022).

1.1.8 Malaria Outbreaks in Zambezi region

Zambezi region has seen increases in malaria cases over the past five years and has overtaken the Kavango regions as being the most malarious region in Namibia with over 80% of cases in Namibia being reported from the region in 2021 and 2022 (NVDCP, 2022). In 2017, 2021 and 2022, Zambezi region experienced malaria outbreaks which may have been due to several causes including population movement leading to importation of malaria, reduced coverage of interventions such as IRS, net distribution and increased rainfall. All these highlight the difficulty in planning for malaria interventions before the malaria season as is currently done by the national malaria program. Molecular tools could be used in season to analyse samples to monitor parameters such as transmission intensity and transmission risk that can inform better deployment of interventions to manage outbreaks. Samples obtained from the 2017 and 2021 outbreaks were utilized to determine if such a use case was possible for the molecular tools. The 2017 and 2021 samples were analysed using molecular tools to assess whether they can help unpack parameters during the course of an outbreak such as transmission intensity and transmission risk.

1.2 Statement of the Problem

Namibia's progress towards malaria elimination has stalled in the last five years due to outbreaks in northern Namibia (Smith et al., 2021). An effective surveillance system is an important intervention to support malaria elimination, as poor surveillance data can prevent implementation of appropriate strategies to contain outbreaks and spreading of transmission foci (Lourenço et al., 2019). Namibia uses RDTs for malaria

case management and surveillance, however, RDTs have been shown to not be sensitive enough to detect all cases at low transmission settings such as Zambezi region resulting in undetected and untreated low density infections that can still perpetuate transmission (McCreesh et al., 2018; Tambo et al., 2018). RDTs can give false-positive results for up to 4 weeks due to antigen persistence, even after successful antimalarial treatment (Agbana et al., 2022). Furthermore, they do not provide quantitative information and do not differentiate between parasite stages (Idárraga et al., 2021) which can assess transmission intensity and risk of transmission. Therefore, there is a need for more sensitive and specific tools for surveillance of malaria. Molecular diagnostic tools are more sensitive and specific than RDTs in the detection of *Plasmodium* parasites. However, it is not known whether they can be used for determining the true infection burden and assess risk of transmission and transmission intensity at low transmission settings, parameters that can be used for better planning of outbreak responses.

1.3 Objectives of the Study

Samples from the 2017 and 2021 malaria outbreak seasons were studied as use cases for malaria molecular surveillance at low transmission settings; to assess transmission intensity and risk based on parasitaemia and gametocytaemia. This study was conducted to:

1. To confirm and determine *Plasmodium spp* from Dried Blood Spots (DBS) from RDT confirmed malaria cases collected during RACD in the Zambezi region during the 2017 and 2021 malaria outbreak season using conventional PCR.
2. To determine the parasite densities from Dried Blood Spots (DBS) collected in the Zambezi region during the 2017 and 2021 malaria outbreak using

quantitative PCR, and whether the detected parasitaemia could be used to predict transmission intensity of the outbreaks.

3. To quantify female *Plasmodium* gametocytes using *Pfs25* transcripts on samples confirmed as *Plasmodium falciparum* during the 2017 and 2021 malaria outbreak season using Reverse Transcriptase qPCR, and determine whether the gametocytaemia could be used as a predictor of risk of transmission of the outbreaks.

1.4 Hypothesis

H_1: Malaria positive RDTs collected during the 2017 and 2021 malaria outbreaks do not give an accurate representation of malaria cases that occurred during the two outbreaks.

H_2: Parasitaemia can be used as an indicator for transmission intensity, high parasitaemia is associated with high malaria transmission intensity than low parasitaemia at low transmission settings.

H_3: High parasitaemia in malaria positive cases contribute to high malaria transmission risk through increased gametocyte carriage during malaria outbreaks at low transmission settings.

1.5 Significance of the Study

Identifying *Plasmodium falciparum* species, quantifying parasite densities and gametocytes during outbreak seasons can provide an indication on transmission risk and intensity. This evidence-based information can therefore guide malaria control programs such as the National Vector-Borne Disease Control Program (NVDCP) and policy makers in understanding the true infection burden as well as the resources required to achieve elimination. The results from this study can contribute to the

malaria elimination efforts in Namibia with evidence-based information to optimize and target malaria intervention by monitoring and verifying progress towards zero local transmission. Therefore, the accurate detection of malaria infections during the outbreak seasons is essential in providing high-quality case management to all malaria infected patients, as well as to inform surveillance systems to allow monitoring progress and guidance on where to target interventions.

1.6 Limitations of the Study

- The study is limited to only Zambezi region, and is not representative of the whole country.
- This study population was based on index cases presenting as Malaria positive by RDTs through passive case detection at the clinic. RDT negative cases were not included as part of the study.

1.7 Delimitations of the study

- The two malaria outbreak seasons focused on, are the 2017 and 2021 Outbreak seasons.
- The study only focused on *Plasmodium falciparum*, for quantification of parasite densities and gametocytes, and not on the other species, since *P. falciparum* is the dominant parasite and the deadliest of the 5 species.
- Only PCR positive *P. falciparum* samples were used to extract mRNA and look at the gametocyte carriage of only female *Pfs25* specific gametocyte transcripts of *P. falciparum*.

Chapter 2. Literature Review

2.1 Malaria, and its Current Status Globally

Malaria is a mosquito-transmitted infectious disease that is caused by protozoan parasites belonging to the genus *Plasmodium* (Patel, Dunican & Cunnington, 2020). Infections in humans primarily involve five *Plasmodium* species, namely: *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae*, and *Plasmodium knowlesi* now considered the fifth species of *Plasmodium* causing malaria in humans (Fitri et al., 2022). *Plasmodium knowlesi* is found in nature in long-tailed and pig-tailed macaques and naturally acquired human infections were thought to be extremely rare until a large number of human infections were reported in 2004 in Sarawak, Malaysian Borneo (Singh & Daneshvar, 2013). From the five, *Plasmodium falciparum* is the most virulent, prevalent and deadliest (Belete, 2020; Patel, Dunican & Cunnington, 2020). According to the latest World malaria report, there were 241 million cases of malaria in 2020 compared to 227 million cases in 2019. The estimated number of malaria deaths stood at 627 000 in 2020 – an increase of 69 000 deaths over the previous year where about two thirds of the deaths (47 000) were due to disruptions during the COVID-19 pandemic (World Health Organization, 2021).

The WHO African Region, with an estimated 228 million cases in 2020 increased from a total of 207 million cases in 2000. The region accounted for about 95% of the malaria cases. Case incidence between 2000-2019 in the WHO African Region reduced from 368 to 222 per 1000 population at risk, but increased to 232 in 2020, mainly because of disruptions to services during the COVID-19 pandemic. The WHO South-East Asia Region accounted for about 2% of the burden of malaria cases globally, malaria cases reduced by 78%, from 23 million in 2000 to about 5 million in

2020 (World Health Organization, 2021). Malaria cases in the WHO Eastern Mediterranean Region reduced by 38%, from about 7 million cases in 2000 to about 4 million in 2015 and between 2016 and 2020, cases rose by 33% to 5.7 million. The WHO Western Pacific Region had an estimated 1.7 million cases in 2020, a decrease of 39% from the 3 million cases in 2000. In the WHO Region of the Americas, malaria cases reduced by 58% from 1.5 million to 0.65 million and case incidence by 67% (from 14 to 5) between 2000 and 2020 (World Health Organization, 2021). In 2016, the WHO targeted 21 countries with the goal of eliminating malaria in them by 2020. Of these 21 countries, Sri Lanka, Paraguay, Algeria, Argentina and Uzbekistan are certified as malaria-free (Burkot et al., 2019).

2.2 Life cycle of the Malaria parasite

The malaria parasite involves a cyclical infection of both humans and female *Anopheles* mosquitoes. When a mosquito inoculates sporozoites (motile, spore-like forms of the parasite) into the human host, they travel to the liver where they first grow and mature into schizonts and rupture releasing merozoites (Venugopal et al., 2020). After the initial replication in the liver defined as the exo-erythrocytic schizogony, the released merozoites infect and evade more red blood cells which undergo asexual multiplication in the erythrocytes. This is known as the erythrocytic schizogony. This infection of the blood stage causes the clinical symptoms of malaria: fever, headache, vomiting, nausea, and general malaise. The ring stage trophozoites in the red blood cells mature into schizonts, which rupture releasing more merozoites, and some parasites differentiate into sexual erythrocytic stages known as gametocytes (Venugopal et al., 2020).

Gametocytes, defined as the sexual precursors of the parasite occur in both male and female forms, and when ingested during a blood meal by a female *Anopheles* mosquito

mature into gametes within the mosquitoes' gut (Baker, 2010; Sato, 2021). The male gametes penetrate the female gametes generating zygotes that become motile and elongated forming ookinetes. These ookinetes invade the midgut wall of the mosquito where they develop into oocysts. The oocysts then grow, rupture and release sporozoites which make their way to the mosquito's salivary glands thus completing the life cycle. The parasites' multiplication in the mosquito is known as the sporogonic cycle (Graumans et al., 2020). When the *Anopheles* mosquito takes a blood meal on another human, it inoculates anticoagulant saliva together with the sporozoites, which migrate again to the liver, thereby perpetuating and completing the malaria life cycle (Capela, Moreira & Lopes, 2019). The infected mosquito carries the disease from one human to another acting as a vector whereas the human host only transmits the parasite to the mosquitoes and not to other humans. In contrast to the mosquito, only the human host suffers from the presence of the parasite and not the mosquito (Patel, Dunican & Cunnington, 2020). Below is a diagram (Figure 8) showing the life cycle of the parasite in detail:

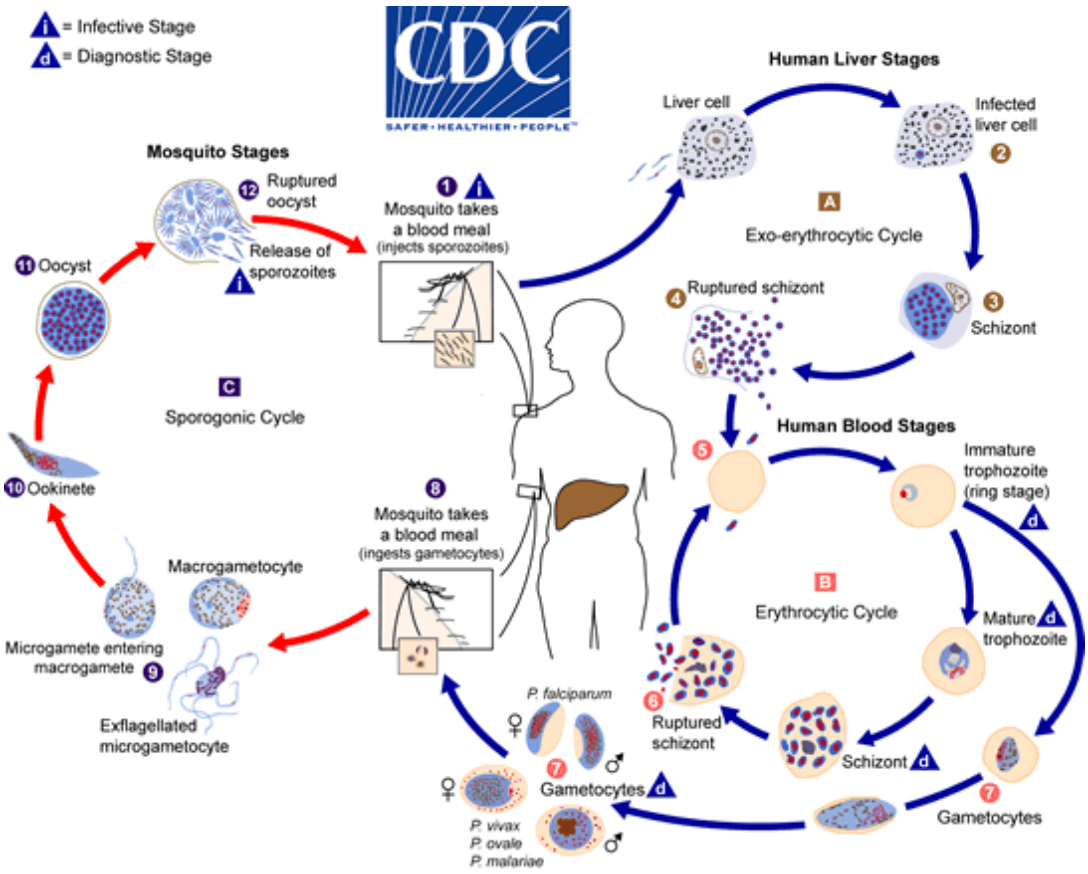


Figure 8: The life cycle of the malaria parasite in both the mosquito vector and human host.

Source: (CDC, 2019) www.dpd.cdc.govt/dpdx

2.3 Malaria Transmission Settings

Transmission settings are defined according to WHO classifications as high transmission being ≥ 35% *Plasmodium falciparum* parasite rate (PfPR) or ≥ 450 cases per 1000 population annual parasite incidence (API). With moderate transmission being between 10–35% PfPR or 250–450 API, and low transmission with 1–10% PfPR or 100–250 API and elimination settings and very low transmission settings being < 1% PfPR but > 0% PfPR or < 100 API (Ashton et al., 2020; Moonasar et al., 2021). High and moderate transmission settings are often found in low-income countries which have low expenditures per person on health care services which

results in weak health systems not easily accessed by the population, lower staff to patient ratios, frequent interruptions of medical supplies and limited use of parasitological diagnosis. In such settings, the primary emphasis of malaria programs has often been on reducing mortality by prevention, appropriate management of severe cases and case reduction (World Health Organization, 2012). In areas of moderate to high transmission, chemoprevention of high-risk groups is recommended which include intermittent preventive treatment of infants (IPTi), intermittent preventive treatment of pregnant women (IPTp) and seasonal malaria chemoprevention (SMC) for children under 5 years with vector control implemented in all malaria-endemic countries. With ACTs used as first-line treatment of uncomplicated cases of *P. falciparum* malaria and intravenous artesunate for severe malaria (Winskill et al., 2020).

Health systems in low-transmission settings are usually stronger than in high-transmission settings (World Health Organization, 2012) as observed with neighbouring Angola. Cross-border migration has been reported from Angola to Namibia to access better healthcare because of poorly equipped and staffed facilities in Angola (Gueye et al., 2014). Malaria may, however, be concentrated in marginalized populations, such as those living in remote border areas, migrant workers and tribal populations, therefore improved and innovative ways may have to be found to reach these groups. In the elimination phase, cases occur sporadically or in distinct foci and imported cases may comprise a significant proportion of all cases and may pose a risk for re-establishment of transmission in areas in which it had previously been interrupted (World Health Organization, 2012).

Depending on the transmission settings, both sub-microscopic gametocytes and microscopically detectable gametocyte densities can cause mosquito infection (Lo et

al., 2015). Malaria programs in low-, moderate- and heterogeneous – settings present unique challenges in that as countries go through epidemiological transition from high to low malaria transition, it becomes challenging to define the point at which an intervention has reached maturity to affect a health outcome. (Ashton et al., 2020). In Namibia, Zambezi Region, transmission is ongoing yet its drivers remain poorly understood. Namibia is a low transmission country located in southern Africa and has experienced a tremendous decline (over 90% decline from 2004) in reported malaria cases over the past decade attributable to the successful implementation of malaria control interventions: IRS, LLINs; use of RDTs, treatment with ACTs; and use of RACD to test and treat those in close proximity to index cases (Jacobson et al., 2019). The incidence levels recorded have categorised Namibia as a low transmission country.

2.4 Challenges in Low Transmission Settings

It is hypothesized that frequent infection in high transmission areas may increase the average parasite density in infected individuals, whereas in lower transmission areas infections may have reached a sub-microscopic phase. However, there is insufficient evidence to support this hypothesis (Lo et al., 2015). In low transmission settings, a substantial proportion of human *Plasmodium* infections are asymptomatic, often remaining undetected by microscopic examination, and these have potential to serve as reservoirs of infection to vector mosquitoes, thus sustaining transmission (Zemene et al., 2018). Therefore understanding the malaria epidemiology in low transmission settings is crucial to design reliable strategies to detect a large reservoir of individuals infected with sub-microscopic infections characterized by low-parasite densities and gametocyte carriage (Tedla, 2019).

2.5 Malaria Control Globally and the Existing Challenges

The goal of global malaria programs is to achieve malaria elimination and eventually eradicate the disease, and achieving this global goal requires first eliminating malaria in individual endemic countries (Coleman, 2022). Malaria control involves a combination of case management which uses diagnosis, treatment and preventative measures using insecticide-treated nets, indoor residual spraying, and intermittent preventive treatment in both pregnant women and children (Deelder et al., 2021). Presently, the World Health Organization has recommended malaria vector control strategies which include bed nets [Figure 9] and IRS [Figure 10] against adult mosquitoes with larval source management (LSM) [Figure 11] as a supplemental intervention. Larval source management (LSM) involves the management of water bodies which harbours the larval stages of mosquitoes to prevent the successful completion of their life cycle (Nalinya, Musoke & Deane, 2022). These three strategies require insecticide use and since 2004, more than 2 billion pyrethroid-treated long-lasting insecticide-treated nets (LLINs) have been deployed and these are attributed to have caused the decline in malaria observed globally (Burkot et al., 2019). Below are figures showing the different vector control interventions: LLINs (Figure 9), IRS (Figure 10), and LSM (Figure 11).

Figure 9

Figure 10

Figure 11

Figure 9: Use of Long Lasting Insecticide-treated nets as an indoor intervention.

Source: (MoHSS, 2022) MoHSS Malaria Program Review 2022

Figure 10: House structure being sprayed with Indoor Residual Spray.

Source: (Nana Kofi Acquah (Photographer) 2017) Malaria TPE Namibia, Novartis Foundation

Figure 11: Larval Source Management as an outdoor intervention.

Source: (MoHSS, 2022) MoHSS Malaria Program Review 2022

Despite the reduction in malaria cases due to these interventions, unintended and inevitable challenges have emerged from insecticide selection pressure from malaria control: vector distributions have changed (Russell et al., 2011), insecticide resistance has emerged and biting patterns (referring to locations and times) have shifted (Russell et al., 2013). There are barriers to uptake of LLINs and IRS which include : a lack of availability, economic costs where free distribution programs are absent, low seasonal use of LLINs during the dry season or other times of the year where it is believed malaria risk is low, alternative uses for the nets including protecting seedlings, as fishing nets and curtains (Nalinya, Musoke & Deane, 2022). Although these interventions provide effective protection when used appropriately, LLINs and IRS have a significant impact on malaria incidence, however due to the mentioned barriers above, it raises questions concerning the direction of malaria prevention efforts with respect to whether policy makers should focus more attention on alternative or complementary interventions (Nalinya, Musoke & Deane, 2022).

Another major challenge faced by southern African countries in attempting malaria elimination is sustained residual indigenous transmission through importation, occurring primarily from higher burden neighbouring countries. Due to the linked countries bordering each other, effective malaria control and eventual elimination in southern Africa can be made possible with consistent regional strategies that

complement and strengthen national control and elimination strategies (Moonasar et al., 2021). The South African Development Community (SADC) has established a regional malaria coordinating organization called the Elimination Eight (E8) [Figure 12] which focuses on building strategies along the borders which include integrated vector management strategy, along with insecticide spraying, the rotation of insecticides and cross-border initiatives (Moonasar et al., 2021). The border of malaria in southern Africa currently runs through the northern provinces of the frontline countries, Namibia, South Africa, Botswana and Eswatini (as shown in figure 12, the dotted line). The second line countries are Angola, Zambia, Zimbabwe and Mozambique. The frontline countries were categorised as countries that drastically reduced malaria and could potentially eliminate the disease by 2020, whereas the second line countries were those that are set to eliminate malaria by 2030. However, the progress achieved in the frontline and second line countries was not furthered or maintained and instead, as a result, elimination efforts have stagnated (Elimination 8, 2018). Below is an image showing the countries bordering Namibia as well as the boundary line of malaria transmission.

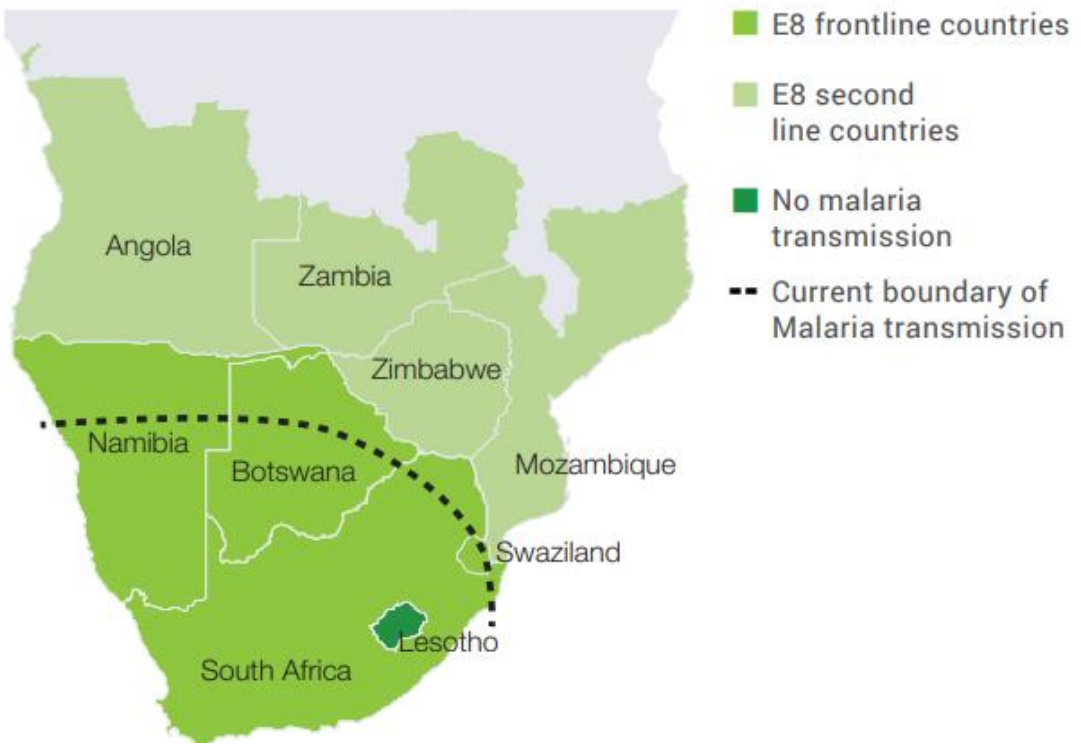


Figure 12: Map illustrating SADC Malaria Elimination 8 countries. Boundary line of malaria transmission cuts across Botswana, Namibia, South Africa and Eswatini, - the four frontline countries.

Source: (Elimination 8, 2018)

These countries aim for the interruption of transmission within their borders, however, the four frontline countries cannot eliminate malaria as long as high transmission remains within the region, and human migratory patterns facilitate parasite importation from regions that have high endemicity (Elimination 8, 2015). Therefore, successful malaria control by the neighbours of these four second line countries (Angola, Zambia, Zimbabwe and Mozambique) can support the elimination strategy of the four eliminating countries, as it serves in reducing the reservoir of potential infection that could cross the border and re-introduce infection in areas aiming to interrupt transmission. As the four frontline countries (previously mentioned) achieve elimination, the interruption of transmission within their boundaries will move the

current boundary of malaria transmission upwards, thus laying the foundation for the second line countries to also achieve elimination. Through this elimination strategy, the boundary of malaria transmission can move further upwards, possibly including additional countries that can be expected to join the original E8 until the region is free of malaria (Elimination 8, 2015).

2.6 Malaria Diagnosis

2.6.1 Different Diagnostic Tools

The WHO recommends parasitological confirmation of malaria positive patients with RDTs or microscopy (Iwuafor et al., 2019; Nundu et al., 2022) showing the presence of the infecting *Plasmodium* parasite. This is important in order to administer the correct treatment in a timely manner, as prolonged exposure of uncomplicated malaria without treatment leads to severe or complicated malaria and eventually death (Mathison & Pritt, 2017; Mousa et al., 2020). Confirmatory parasitological diagnosis is also important to avoid overtreatment with artemisinin-based combination therapy (ACTs) which may lead to the progression of resistance and waste of resources (Tedla, 2019). The early and accurate diagnosis of malaria is essential for both effective disease case management and malaria surveillance (Berzosa et al., 2018). Improved diagnosis and modern intervention tools have reduced the malaria burden, however, this disease still causes high mortality every year (Dhiman, 2019). The search and use of sensitive and specific diagnosis of malaria becomes increasingly important as malaria prevalence in many regions continues to decline (Robinson et al., 2019). In low transmission settings with few infected individuals in the community, molecular-epidemiological studies require screening of large numbers of samples with high sensitive diagnostic tools to identify the remaining infected individuals (Grossenbacher et al., 2020). Therefore, there is a need for more sensitive molecular

techniques that amplify parasite DNA as malaria eradication efforts seek to eliminate asymptomatic, sub-microscopic infections that serve as reservoirs for transmission that become carriers and constitute a source of infection for others (Niang et al., 2017; Grossenbacher et al., 2020).

Available malaria diagnostic tools for identification of *Plasmodium* species in human blood samples include microscopy, regarded as a gold standard method, immuno-chromatographic lateral flow assays, serology, nucleic acid amplification techniques (NATs) which include polymerase chain reaction (PCR) and isothermal amplification (LAMP) (Shibeshi, Kifle & Atnafie, 2020). Currently available immuno-chromatographic lateral flow rapid diagnostic tests and field microscopy recommended by the WHO are unlikely to consistently detect low density infections making them insufficiently sensitive for detecting all carriers. However, molecular diagnostic platforms, such as PCR and LAMP, are currently available in reference laboratories, and have high sensitivity and specificity, but have high costs both financially and in turnaround time (Britton, Cheng & McCarthy, 2016).

2.6.2 Clinical Diagnosis and Symptoms

Clinical diagnosis is based on the patient's travel history, symptoms, and the physical findings at examination. The first symptoms of malaria include most often: fever, chills, sweats, headaches, muscle pains, nausea and vomiting (Tangpukdee et al., 2009). These symptoms can be non-specific as the signs and symptoms can overlap with other common febrile illnesses (Nega et al., 2020). Symptoms, or phenotypes of severe or complicated malaria include: severe malarial anaemia, cerebral malaria, and respiratory distress syndrome (Vandermosten et al., 2018; Mousa et al., 2020).

2.6.3 Parasitological Diagnosis

2.6.3.1 Microscopic diagnosis

Microscopy is a relatively simple technique that any laboratory that carries out haematology tests can perform. Microscopy is still considered the "gold standard" for malaria diagnosis in endemic countries, as it is inexpensive, allows parasite detection, parasite identification, parasite quantification, is useful during follow-up of parasitaemia clearance and allows the possible identification of other pathogens (Berzosa et al., 2018; Dhorda et al., 2020; Id et al., 2020; Horning et al., 2021; Idárraga et al., 2021). Malaria parasites can be identified under the microscope by examining a drop of the patients' peripheral blood as either a thin or thick stained smear [Figure 13]. The blood is spread as a thin and thick blood smear and stained with a Romanowsky stain, most often Giemsa stain for malaria, and is examined with a 100X oil immersion and the ocular lens, giving a total of 1000X magnification (Dhorda et al., 2020). Thin and thick blood films have distinct uses with thick films typically used for diagnosis and for quantification of low parasitaemia infections, because the larger blood volume gives a lower limit of detection (LOD) and more stable parasite counts. Thin films are used for species identification and for quantification of high parasitaemia infections (Delahunt et al., 2019). Accurate parasite quantification is essential for the management of malaria patients by assessing disease severity associated with high parasitaemia, and for testing antimalarial drug resistance as well as monitoring drug-efficacy (Das et al., 2021). These are vital pieces of information that guide what treatment to be administered to the patient.

In many malaria-endemic regions, microscopic diagnosis has certain limitations, including a shortage of skilled microscopists, inadequate quality control, and the possibility of misdiagnosis due to low parasitaemia or mixed infections (Berzosa et

al., 2018). As such the training of microscopists and regular competency assessment is critical in diagnosis of malaria to ensure the required microscopy skills are not lost over time. Microscopy requires material resources which can lead to high variability in the quality of microscopy, which could potentially hinder programmatic and research operations in the malaria-endemic regions (Horning et al., 2021; Bachman et al., 2022). Despite these setbacks, microscopy remains an essential tool to support clinical research, severe malaria case management and monitoring of antimalarial treatment efficacy (Dhorda et al., 2020). Below is a figure which shows a thick and thin smear and red blood cells infected with *Plasmodium falciparum*, as visualized under the microscope.

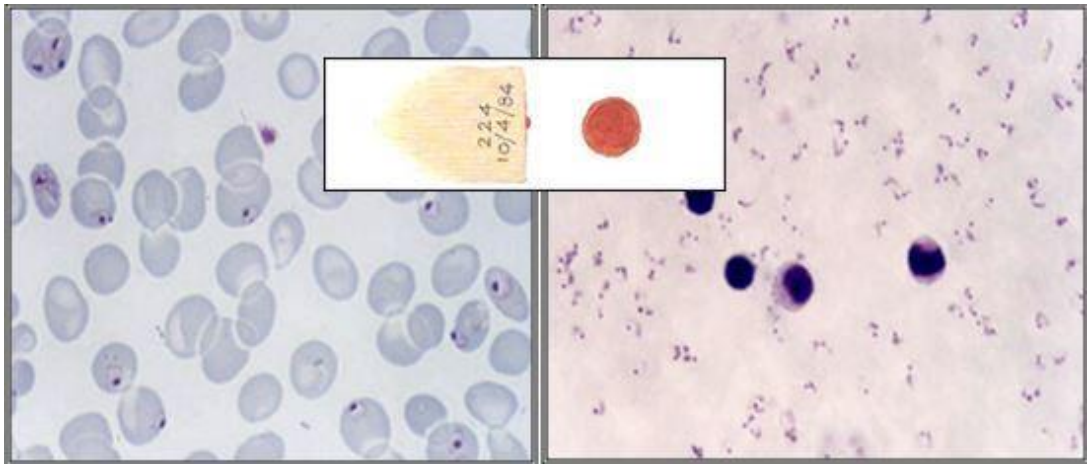


Figure 13: Blood sample on microscopic slide with a thick smear (right) and thin smear (left), and how the red blood cells look like when infected with *Plasmodium falciparum.*

Source: (Tankeshwar, 2022)

2.6.3.2 Antigen Detection by Rapid Diagnostic Tests

The limitation of microscopy prompted research and development of reliable, easy-to-perform rapid diagnostic tests (RDTs) to detect the presence of malaria parasites. Malaria RDTs are a simple, rapid, cheap and are a field-deployable way to identify a malaria infection at the point-of-care, being especially useful in low-resource and rural settings without access to laboratory facilities that require stable electricity (Galatas et al., 2020). Therefore, in the absence of well-prepared technicians for microscopic diagnosis in many areas of sub-Saharan Africa, the WHO has recommended the use of RDTs as a good alternative method for malaria diagnosis (Ugah et al., 2017). In remote and rural parts of sub-Saharan Africa where microscopy is inaccessible/unavailable, RDTs have become the primary tool for the parasitological diagnosis or confirmation of malaria, as they allow for rapid diagnosis with minimal technical skills (Kozycki et al., 2017; Mudare et al., 2021).

Rapid diagnostic tests (RDTs) are lateral flow immuno-chromatographic antigen-detection tests, which rely on the capture of dye-labelled antibodies to produce a visible band on a strip of nitro-cellulose encased in plastic housing, called a cassette (Gillet et al., 2009; Mouatcho & Goldring, 2013; Oyegoke, Maharaj, et al., 2022). RDTs function by detecting proteins released from parasitized red blood cells which include histidine rich protein (HRP2), *Plasmodium* lactate dehydrogenase (pLDH) and aldolase (Ugah et al., 2017) although the pLDH and aldolase RDT are less sensitive compared to tests that detect HRP2 (Hopkins et al., 2008).

RDTs are limited mainly by their inability to detect infections at low parasitaemia (Apinjoh et al., 2021). They have poor performance in terms of sensitivity for low density parasite infections which limits their utility in elimination settings, as their detection limit is at approximately 100–200 p/μL (Das et al., 2018). Also, the

performance of the histidine rich protein 2-immuno-chromatography-based RDTs are affected by the detection of persistent antigenicity from previous infections. They are unable to distinguish between past and current infections due to antigen persistence (Oboh et al., 2021), which can potentially lead to false-positives and overestimate prevalence (Apinjoh et al., 2021; Manjurano et al., 2021). RDTs have an inability to quantify parasitaemia and differentiate parasite stages (Idárraga et al., 2021). Other disadvantages are false-negative RDTs which can arise for different reasons, such as very low or high parasitaemia, dysfunctional tests due to non-functioning antibodies or test equipment after improperly stored test kits, as RDTs' major constraint is degradation by extreme temperatures and humidity (Barbé et al., 2012; Schlabe et al., 2021). According to several national guidelines, each RDT result needs confirmation by microscopy and/or qPCR, a strategy that can potentially prevent cases with HRP2-deletions being overlooked (Schlabe et al., 2021).

Malaria cases in Namibia are reported based on passively detected cases confirmed by RDT at clinics and by tracing secondary cases through tracking a passive case back to their homestead with the Carestart™ Malaria HRP-2/pLDH (Pf/pan) Combo rapid diagnostic test (Smith, Auala, Haindongo, et al., 2017). Despite the numerous advantages that RDT offers, microscopy still remains the reference standard method in malaria-endemic regions (Bamou et al., 2021). Below is a figure that shows the RDT mechanism.

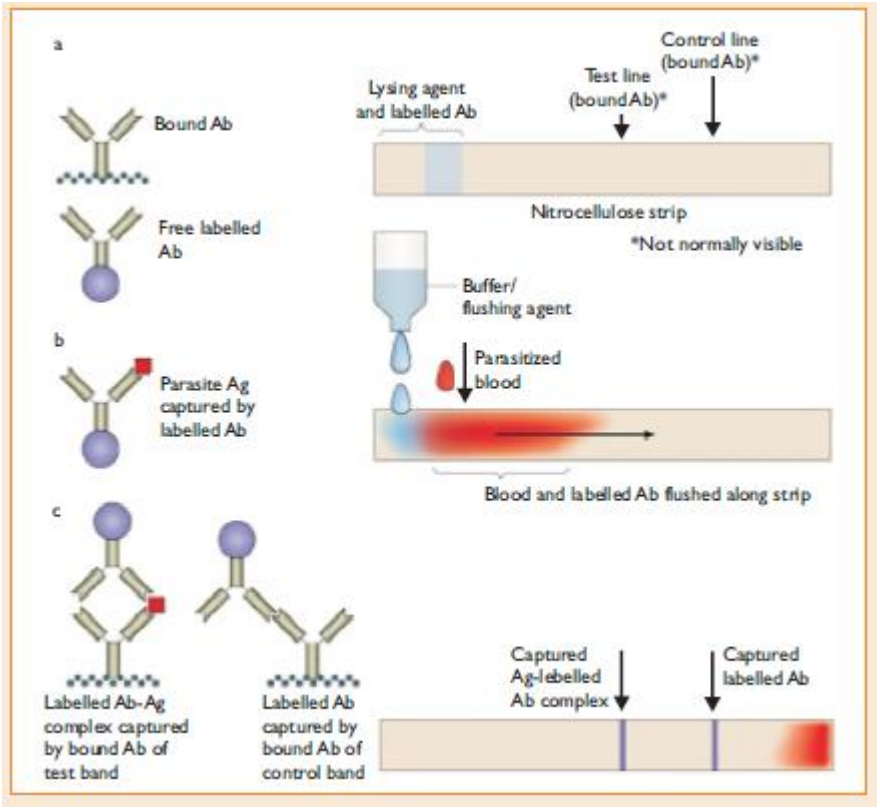


Figure 14: Illustration of the Malaria Rapid Diagnostic Test Mechanism.

Source: (PathLabs, 2022)

2.6.4 Molecular Diagnosis

2.6.4.1 Polymerase Chain Reaction (PCR)

The Polymerase Chain Reaction is a molecular tool that can be used for the diagnosis of malaria, which includes different types of PCR assays, such as conventional PCR, nested PCR, qPCR, and multiplex PCR (Tedla, 2019). PCR in general is a very sensitive molecular diagnostic modality that is capable of detecting and identifying asymptomatic, sub-microscopic infections (Britton, Cheng & McCarthy, 2016). Although microscopy remains the "gold standard" diagnostic test for malaria in clinical settings (Murray et al., 2008; Berzosa et al., 2018; Idárraga et al., 2021; Mudare et al., 2021), polymerase chain reaction (PCR)-based assays are the most sensitive methods available. They are able to detect parasitaemia as low as 2–

5 parasites/μL (Berzosa et al., 2018; Tedla, 2019; Idárraga et al., 2021), whereas, RDTs and microscopy have a detection limit of 100-200 parasites/μL (Tedla, 2019; Picot, Cucherat & Bienvenu, 2020). Quantitative PCR (qPCR) is important for quantifying parasite density, thus the sensitivity of PCR for identifying low level parasitaemia has been shown to be improved by performing qPCR which has been shown to have a limit of detection (LOD) of 22 parasites/mL (Imwong et al., 2014).

The WHO recommends that nucleic acid amplification tests be considered only for epidemiological research and survey mapping of sub-microscopic infections (Berzosa et al., 2018). However, implementing molecular techniques as diagnostic methods in sub-Saharan Africa is complicated as they are not appropriate for use in the field. This is due to the expensive equipment required, high cost of reagents, qualified personnel required as well as need for constant, stable power supply (Britton, Cheng & McCarthy, 2016; Berzosa et al., 2018). Therefore PCR is still limited to well-equipped centres that are mostly far from remote endemic areas, which is not ideal for quick commencement of treatment for malaria cases (Picot, Cucherat & Bienvenu, 2020). These drawbacks have limited PCR-based diagnostic assays primarily to research settings (Smith, 2010).

2.6.4.2 Loop-Mediated Isothermal Amplification (LAMP)

There are three isothermal amplification techniques, namely LAMP, Rolling Circle Amplification, and Clustered Regular Interspaced Short Palindromic Repeat systems (Boonbanjong et al., 2022). These have been continuously developed for disease diagnosis. Among these three isothermal amplification systems, LAMP has been widely used for Point-of-Care applications because of the short amplification times (Boonbanjong et al., 2022). Loop-mediated isothermal amplification (LAMP) of DNA is a molecular technique for nucleic acid amplification to determine the presence of

Plasmodium parasites (Lau et al., 2016; Selvarajah et al., 2020) and was first described in 2000 by Notomi et al., (2000) (Notomi et al., 2000). LAMP is an isothermal molecular method which enables isothermal amplification of the target gene. The LAMP assay uses a DNA polymerase obtained from *Bacillus stearothermophilus*, which has strand displacement activity leading to DNA auto-cycling without temperature changes (Sattabongkot et al., 2014; Picot, Cucherat & Bienvenu, 2020). The different LAMP tests that are available mostly target the mitochondrial genome of the *Plasmodium* parasite and can be performed at a single temperature with a simple heating block or water bath. This reduces the need for laboratory facilities and need for sensitive and expensive machinery such as PCR machines. (Morris & Aydin-Schmidt, 2021). DNA amplification by LAMP can be observed by visual detection by noticing a change in turbidity caused by white precipitate of magnesium pyrophosphate formed during the reaction using the negative and positive controls as references by fluorescence under ultraviolet (UV) light (Sattabongkot et al., 2014). Visual detection avoids the need for opening the reaction tube post-amplification, hence any risks of contamination is reduced because the reaction is conducted in a closed system (Morris & Aydin-Schmidt, 2021).

A study by (Tambo et al., 2018) showed that LAMP and PCR both amplify the detection signal (parasite DNA), and LAMP detected even more positive samples than PCR. LAMP is not affected by most PCR inhibitors (robust) which could explain why it picked up more positive samples than PCR. LAMP is cheaper to set up in terms of machinery and requires minimal training, making it more accessible than PCR in lower resource settings (Tambo et al., 2018). The improved analytical sensitivity of LAMP, resulting in the detection of significantly more infections compared to RDT or microscopy and the reduced time to-result compared to PCR has put LAMP forward

as a promising tool for near point-of-care detection of low parasite density infections in asymptomatic carriers (Morris & Aydin-Schmidt, 2021), especially in low endemic and pre-elimination areas. However, despite its improved analytical sensitivity, LAMP may still miss a substantial proportion of the asymptomatic reservoir of very low-density infections especially in low transmission settings, as studies conducted in asymptomatic and low-density infections have shown a reduced sensitivity when parasite densities go below two parasites per microlitre (Morris & Aydin-Schmidt, 2021).

It is unlikely that LAMP will replace conventional diagnostic tools such as RDTs and microscopy in the point-of-care diagnosis of clinically symptomatic malaria in endemic settings, because LAMP in its current formats does not completely fulfil the World Health Organization (WHO) ASSURED criteria. This criterion includes: being affordable, sensitive, specific, user-friendly, robust and rapid, and equipment free (Mbanefo & Kumar, 2020). In addition, the technicians running the assays require sufficient knowledge to manage DNA contamination, as this is an issue that all nucleic acid amplification-based methods face. Another issue is the high cost of these commercially available assays that act as a limiting factor in commercialising this tool as a point-of-care diagnostic tool. Nevertheless, further improvements of the available commercial kits and detailed assessments of cost-effectiveness could improve the uptake of LAMP on a larger scale (Morris & Aydin-Schmidt, 2021).

2.7 Malaria Treatment

Once the diagnosis of malaria has been made, appropriate antimalarial treatment must be initiated immediately as malaria can progress into a severe, potentially fatal disease when not treated especially when caused by *Plasmodium falciparum* (Marrelli & Brotto, 2016). Malaria is treated with prescription drugs to kill the parasite.

Antimalarial treatments are guided by three main factors: the infecting species of *Plasmodium*, the clinical condition of the patient, and the susceptibility of the parasites to the antimalarial drugs determined by the geographic area where the infection was acquired as well as the previous use of the medicines (Marrelli & Brotto, 2016).

The types of drugs and the length of treatment varies depending on the type of malaria parasite one is infected with, the severity of the symptoms, age, area where infection was acquired, drug resistance status of the parasite, pregnancy status, history of drug allergies or other medications taken by the patient. Malarial treatment is based on natural products, semi-synthetic, and synthetic compounds developed since the 1940s (Belete, 2020). The existing antimalarial drugs are agents grouped into three main classes, namely: quinoline, antifolate, and artemisinin derivatives. There is no single drug that has been identified, found or manufactured that can wipe out all strains of the *Plasmodium* species, and thus, to fight malarial infection efficiently, the drugs are frequently used in combination (Belete, 2020). Resistance to antimalarial medicines threatens global efforts to control and eliminate malaria and this resistance originates from *Plasmodium* genetic mutations that increase in frequency over time (Shibeshi, Kifle & Atnafie, 2020; Deelder et al., 2021).

2.7.1 Artemisinin based Combination Therapy

Artemisinin was first extracted from a plant called *Artemisia annua*, Artemisinin and its derivatives including, artemether, dihydroartemisinin, arteether, and artesunate have broad-spectrum activity and inhibits all parasite phases within erythrocytes, particularly at the early phase of their development (Khanal, 2021). Artemisinin and its derivatives are effective against chloroquine and mefloquine-resistant strains, they are safe, potent, and are rapid-acting blood schizonticides against

all *Plasmodium* species. Artemisinin does not clear the latent liver stages of the parasite, as it has a short half-life and poor bioavailability which leads to drug resistance, making them ineffective as a monotherapy. Therefore, Artemisinin derivatives are recommended for treatment in combination with other antimalarial agents (Belete, 2020). WHO guidelines and MoHSS Malaria Case management guidelines recommend Artemisinin Combination Therapy, Artemisinin and its derivatives with lumefantrine – Artemether lumefantrine (AL) and artesunate-mefloquine as first-line treatments for malaria (World Health Organization, 2015). Recently, resistance to artemisinin has been reported in the form of delayed parasite clearance in Southeast Asia, which poses a threat to the current first-line artemisinin-based combination therapy (Deelder et al., 2021; Jagannathan, 2022).

2.7.2 Primaquine

Primaquine is an 8-aminoquinoline antimalarial drug first synthesized in 1946 which acts as a gametocytocidal drug known to significantly reduce malaria transmission (Ashley, Recht & White, 2014). Despite highly efficient clearance of asexual stage parasites and early gametocytes with ACTs, ACTs do not affect mature *Plasmodium falciparum* gametocytes (Stepniewska et al., 2022). Since mature gametocytes are responsible for onward transmission from humans to mosquitoes and remain unaffected by ACTs, they can persist for several days and even weeks after ACT administration. These individuals treated with ACTs only can continue to be a source for mosquito infections (Stepniewska et al., 2022), therefore Primaquine is recommended, however, this drug induces dose-dependent acute haemolytic anaemia in individuals with glucose-6-phosphate dehydrogenase (G6PD) deficiency (Mwaiswelo et al., 2022). This has led to a limited use of the drug especially in Africa where the condition is common. Due to this issue, the WHO recommends a single low

dose of Primaquine (0.25 mg/kg) in combination with artemisinin-based combination therapies as *P. falciparum* gametocytocidal without the need for prior screening of G6PD status. Studies conducted in Sub Saharan African countries support the safety of this single low dose even in individuals with G6PD deficiency (Mwaiswelo et al., 2022).

2.7.3 Malaria Vaccines

After more than four decades of research and clinical trials for a malaria vaccine, the World Health Organization has recommended the malaria vaccine RTS, S as the first malaria vaccine for widespread use among children living in malaria-endemic areas (Yin, Yan & Li, 2022; Zavala, 2022). The RTS,S vaccine is a pre-erythrocytic *Plasmodium falciparum* malaria vaccine which targets the clinically silent sporozoite and liver stages which aims to eliminate the parasite during its early stages and is developed for routine immunization of young children living in endemic countries (Praet et al., 2022).

The implementation and the safety, effectiveness and impact evaluation of RTS, S in a real-life setting is a complex undertaking that requires the establishment of large-scale and strong partnerships. Also assessing the benefit-risk profile of a 4-dose primary schedule vaccine in low and lower-middle income countries requires the establishment of ad-hoc tools and quality control methods to allow collection of robust and reliable data (Praet et al., 2022). Whilst technical and operational expertise is key to achieve this goal, human and financial resource needs should not be underestimated. The experience with RTS, S in sub-Saharan Africa highlights important aspects to be considered when planning and implementing a vaccine post-approval plan in low and lower-middle income countries. The RTS, S experience will pave the way for the development and implementation of new generation malaria vaccines, and of other

vaccines (Praet et al., 2022). In October 2021, the World Health Organization recommended the widespread use of the RTS, S malaria vaccine for children at risk in sub-Saharan Africa and other regions with moderate to high transmission of malaria caused by *Plasmodium falciparum* (Egbewande, 2022). Mosquirix has paved the way for a more potent R21 vaccine (has reached the WHO-specified goal of 75% or greater efficacy over 12 months) developed by the University of Oxford, with evidence showing vaccine effectiveness at as much as 80% and has currently progressed to a phase 3 licensure trial (Datoo et al., 2022). R21 is a better variant of the RTS, S that is a subunit antimalarial vaccine and is considered the next generation antimalarial vaccine to prevent malaria (Nadeem et al., 2022). Below is a table with the different vaccines and their phase statuses.

Table 1: Pre-erythrocytic, blood-stage and transmission-blocking vaccines, their immunogen type and phase status.

Source: (Duffy & Patrick Gorres, 2020)

Vaccine Candidate	**Immunogen type**	**Current status**
Pre-erythrocytic stage (anti-infection)	***Pre-erythrocytic stage (anti-infection)***	***Pre-erythrocytic stage (anti-infection)***
RTS,S	Subunit	Phase 4
R21	Subunit	Phase ½
Full-length CSP	Subunit	Phase 1
PfSPZ Vaccine	Whole sporozoite (radiation attenuation)	Phase 2
Chemoprophylaxis vaccination (CVac)	Whole sporozoite (chemical attenuation)	Phase 2
Genetically attenuated parasite (GAP) vaccines	Whole sporozoite (genetic attenuation)	Phase 1
Blood stage	***Blood stage***	***Blood stage***
PfRH5	Subunit	Phase 1
AMA1-RON2	Subunit	Preclinical
PfSEA-1	Subunit	Preclinical
PfGARP	Subunit	Preclinical
Chemically attenuated parasite (CAP) vaccines	Whole blood-stage parasite	Phase 1
VAR2CSA (Placental malaria)	Subunit	Phase 1
PvDBP (*Plasmodium vivax*)	Subunit	Phase 1
Mosquito stage (Transmission-blocking)	***Mosquito stage (Transmission-blocking)***	***Mosquito stage (Transmission-blocking)***
Pfs25	Subunit	Phase 1
Pfs230	Subunit	Phase 2
Pfs48/45	Subunit	Preclinical
Pvs230 (*Plasmodium vivax*)	Subunit	Preclinical

2.8 Malaria Molecular Surveillance

Malaria surveillance is defined as the continuous and systematic collection, analysis and interpretation of epidemiological data. This is the core monitoring and evaluation tool for malaria control programs, and provides the framework for effective allocation of resources (Guirou et al., 2020). A critical surveillance measure, which closely reflects malaria transmission intensity, is the parasite rate; defined as the proportion of the population found to carry parasites in their peripheral blood (Guirou et al., 2020).

Different LAMP technologies have been developed with sensitivity as low as 1 to 10 parasites/µL. PCR-based techniques are more sensitive, increasing the sensitivity up to 0.022–0.1 parasites/µL and 0.016 parasites/µL by RTqPCR (Nsanzabana, 2019). However, these techniques are better suited for well-equipped laboratories, with well-trained staff. In Malaria, molecular surveillance can be used to monitor trends of disease burden and impacts of interventions and to determine sources of parasites in eliminating areas. Case detections in malaria elimination settings are still mainly based on microscopy and RDTs. Even though these techniques have proved to be successful in some malaria elimination settings, the addition of molecular techniques could greatly improve the sensitivity of parasite detection, especially for asymptomatic cases during passive case detection (PCD), active case detection (ACD), and reactive case detection (RACD) for surveillance (Nsanzabana, 2019; Naing et al., 2022).

Molecular techniques such as QT-NASBA and RTqPCR have the advantage of detecting gametocytes and can be used in studies evaluating gametocytocidal drug efficacy, or to evaluate the impact of interventions aiming at reducing transmission by looking at risk of transmission (Nsanzabana, 2019). These different interventions do not require prompt results for case management but the analysis conducted in a sub-

national or national reference laboratory can provide more accurate, quality data that give a reflection of the true malaria burden looking at transmission intensity as well as risk of transmission, and can guide malaria control programs with evidence-based information (Nsanzabana, 2019).

2.8.1 Parasite Density as an Indication of Transmission Intensity

The level of infection, expressed as parasite density is defined as the number of asexual forms of parasite relative to a blood volume such as microlitre or a percentage of white blood cells (Hammami, Nuel & Garcia, 2013). Parasite density can be estimated using microscopy by counting the number of asexual parasites against white blood cells or per oil immersion field on a thick smear or against red blood cells on a thin smear (Das et al., 2021). Estimating the level of parasitaemia in the thick blood smear examination can be calculated by using a simple mathematical formula which is: Number of parasites x 8,000 / Number of white blood cells (200) (Padilla-Rodríguez, Olivera & Guevara-García, 2020). Even though microscopy remains the gold standard for malaria diagnosis and quantification, more sensitive and robust diagnostic tools such as PCR are used in research settings to monitor interventions and track sub-microscopic infections due to some of the drawbacks of microscopy and RDTs. Monitoring changes in malaria transmission requires detailed and complete data on malaria infection as well as the disease burden, human, mosquito and other host distributions and wider environmental factors. The WHO recognizes surveillance as a core intervention required to achieve malaria elimination (Fornace et al., 2021). Therefore, more sensitive molecular tools such as qPCR are used for both detection and quantification of parasite DNA which requires the use of labelled probes for increased specificity (Oboh et al., 2021).

Defining parasite density levels is important for evaluating the potential severity of the disease, the initiation of etiological treatment and monitoring of the therapeutic response (Padilla-Rodríguez, Olivera & Guevara-García, 2020). Hyperparasitaemia has been listed as one of the criterion of severe *P. falciparum* malaria by the World Health Organization. There are previous studies that show that there is a correlation between parasite density and severity of malarial infections and that mortality is also correlated with the degree of parasitaemia as patients with the highest parasite densities have been found to have the highest fatality rates (Kotepui et al., 2015). Additionally, high parasitaemia due to *Plasmodium falciparum* infection can lead to severe anaemia. It has also been noted by previous studies that increasing levels of *P. falciparum* parasite loads result in decreased platelet counts (Kotepui et al., 2015). Therefore, quantifying true RDT positive parasite densities can potentially provide a reference point on the different outbreak seasons 2017 and 2021. The following are Parasitaemia case definitions and are categorized as low (< 1000 parasites/μL blood), moderate (1000–4999 parasites/μL blood), high (5000–99,999 parasites/μL blood), and hyperparasitaemia (≥ 100,000 parasites/μL blood) (Chipwaza & Sumaye, 2020) and these case definitions were used to categorize the parasitaemia analysed in this study.

The density of malaria parasites in infected human blood can range from below 1 parasite/μL to tens of thousands of parasites/μL (Koepfli et al., 2016). Malaria control programs require an understanding of parasite densities and their distribution in the general population in order to estimate severity of the disease in the individual and the proportion of infections below the limit of detection of field-deployable diagnostic tools such as microscopy or rapid diagnostic tests. Parasite densities have been determined by counting parasites by microscopy (Koepfli et al., 2016), however due

to the limited amount of blood examined, parasites are only reliably detected if their density is above 50–100 parasites/μL, which is a drawback to using microscopy and RDTs (Siahaan, 2018). Absolute quantification of parasites by qPCR is however challenging as: a standard curve must be generated, either from plasmids containing the target sequence of the qPCR, or from cultured ring-stage parasites counted by microscopy. Currently the absence of reference standard curves makes comparison of qPCR results across laboratories difficult (Koepfli et al., 2016).

2.8.2 Gametocyte Carriage as an Indicator of Transmission Risk

Asexual parasite density is an important predictor for the probability of detecting gametocytes, and in many cases, it can explain patterns in gametocyte carriage (Koepfli & Yan, 2019). Gametocytes do not replicate in the blood, so their quantity depends solely on the number of progenitor asexual stages in the bloodstream (Kosasih et al., 2021). Gametocytes are the precursors of male and female gametes of malaria parasites, that is, they are the sexual forms of the parasite and are transmitted from the human host to the mosquito vector during a blood meal (Roberts et al., 2013; Guiguemde et al., 2020). They develop in the human host during the developmental switch from asexual replication in the red blood cells (Venugopal et al., 2020). Gametocytes assure the continuity of the malaria parasite life cycle in *Anopheles* mosquitoes and although crucial for the understanding of the infectivity cycle and disease transmission, the ultimate and proximate mechanisms leading to gametocyte differentiation is not fully understood (Sondo et al., 2021). Gametocyte conversion rates might vary, depending on factors such as the level of acquired immunity, or as a reaction to treatment. When conditions in the human host deteriorate, or when mosquito densities increase for example in regions of seasonal transmission, a higher investment in transmission would increase survival of the parasite. Under controlled

laboratory conditions, higher proportions of gametocytes were found after sub curative drug treatment, however it remains unclear whether parasites in the field adapt the gametocyte conversion rate in response to external factors to an extent that it is measurable in population-wide studies. A better understanding of commitment to transmission could help control programs to target gametocytes specifically through drugs and transmission-blocking vaccines (Koepfli & Yan, 2019).

The clinical symptoms of malaria are largely the result of the replication of asexual stages in human blood called merozoites, however, the gametocytes are not responsible for clinical manifestations, as they only serve in mediating transmission from humans to mosquitoes (Meibalan & Marti, 2017). Due to this, the prevalence of gametocytes is therefore used as a parameter for assessing malaria transmission (Bousema et al., 2004; Guiguemde et al., 2020). Gametocytes are distinct from their asexual blood stage counterparts and this can be reflected in their distinct patterns of gene expression, cellular development as well as the metabolism (Baker, 2010). Malaria-infected individuals may harbour gametocytes below the detection threshold of the recommended conventional tools, RDTs and microscopy, but can be detected and amplified by molecular techniques that target the (ribonucleic acid) RNA transcripts of gametocyte-specifically expressed genes (Wampfler et al., 2013). In most studies, in order to limit the number of RNA extractions and RTqPCRs, only infections that test positive for parasites by DNA-based PCR are screened for gametocytes (Koepfli & Yan, 2019), and this was done for this study, only the samples that were positive by PCR were used for RNA extractions and quantifications.

The presence of gametocytes in the circulation of infected individuals is imperative for malaria to remain endemic in a given community (Cox, 2010; Kuamsab et al., 2012). In a number of malaria-endemic countries, transmission is limited to a brief wet

season, and absent during the dry season. *Plasmodium falciparum* infections persist at low densities during the dry season and initiate transmission once mosquitos become abundant in the wet season. It has been speculated that parasites might increase gametocyte conversion at the beginning of the transmission season when mosquito populations are higher for onward transmission. However when it is dry, a parasite could benefit from not producing gametocytes during the dry season as resources invested in gametocyte development can be potentially lost if no mosquitos are present and thus no chance for onward transmission (Koepfli & Yan, 2019).

The effect of sex ratio on transmission success may depend on total gametocyte density, in low-density infections, a larger investment in male gametocytes is favourable to increase the chance that all females are fertilized (Tadesse et al., 2019). Male gametocytes can produce up to eight microgametes upon activation and female-biased gametocyte sex ratios contribute to a more balanced number of macro- and microgametes in the mosquito mid gut (Henry et al., 2019; Munro & McMorran, 2022). At high gametocyte densities, a male-biased sex ratio leads to less efficient transmission favouring a less male-biased ratio. Recent studies show that molecular gametocyte sex ratio assays supported the density dependency of sex ratio with a higher proportion of male gametocytes in low-density infections (Tadesse et al., 2019). The same study also suggested that quantifying male and female gametocytes allowed a better prediction of mosquito infection rates as compared to previous estimates that quantified female gametocytes only and that the number of male gametocytes may become a limiting factor in determining transmission success at low densities. If male gametocytes are indeed a limiting factor for transmission, diagnostics that quantify male gametocytes might be better indicators of transmission potential than female gametocyte diagnostics that are currently most widely used (Tadesse et al., 2019).

Contrasting predictions can be made regarding the relationship between gametocyte production and multiplicity of infection (MOI), such as the existence of within- host competition among distinct genotypes of malaria parasites could result in an increased investment in reproduction and transmission to escape the competition. However, in the midst of competition, parasites could divert resource away from transmission and towards replication to maximize their competitive success and hence their within-host survival (Sondo et al., 2021). Other studies have shown that MOI can promote the longevity of *P. falciparum* infection in patients and their ability to produce gametocytes, hence a positive relationship between MOI and gametocyte carriage has been reported in previous studies and can translate into a positive correlation between MOI and mosquito infection rates (Sondo et al., 2021). Being able to identify prognostic indicators of gametocyte carriage contributes to the successful implementation of interventions aimed at reducing transmission (Muthui et al., 2019). Therefore, having knowledge on gametocyte carriage in a population is important to accurately assess the level of parasite transmission leading to an understanding of the transmission risk (Guiguemde et al., 2020). Finding strategies and transmission-blocking interventions that interfere with gametocyte development or persistence could result in a reduction of malaria transmission (Bousema et al., 2004).

Chapter 3. Research Methods

3.1 Research Design

This study used a qualitative design for confirming *Plasmodium* species and a quantitative design for parasite and gametocyte density as measures of transmission intensity and risk while comparing these parameters for the outbreaks in two malaria seasons, 2017 and 2021. Individuals residing in close proximity to the positive case (index) were tested and if found positive were treated according to MoHSS malaria treatment guidelines.

3.2 Procedure

3.2.1 Sample Collection and Study Site

Zambezi region is located towards the north-eastern part of the country and contributed to over 80% of the recorded malaria cases. Individuals who tested positive for malaria during the malaria seasons 2017 and 2021 (February to May) using RDTs through passive case detection at the Choi and Sesheke health facilities in Zambezi region, were classified as an index case. The index cases were traced back to their homestead using RACD which is a surveillance tool used by the MoHSS to identify and treat secondary malaria cases in close proximity to the households of index cases. Index cases were traced back to the homesteads within 48 hours, all persons who have spent a night within the index case household and in neighbouring households within 100 m radius (Nghipumbwa et al., 2018) of the index case were tested for malaria since malaria cases tend to be geographically clustered (Stresman et al., 2012; Smith, Auala, Tambo, et al., 2017; Mukaka et al., 2021). Malaria RDTs are conducted as the standard of care as per MoHSS, and DBSs were collected by a registered nurse from all individuals (both index and secondary) who were diagnosed as being malaria positive by RDTs. According to MoHSS treatment guidelines, if a patient had a history

of infection, they are not treated, but for this study they were tested and a DBS was collected when found positive for molecular surveillance. History of infection can be confirmed by looking at the patients' health passport, which prevents unnecessarily over treating the patient. In addition to health passports that show a history of malaria, all persons who were part of RACD and were positive for malaria (secondary cases), a case investigation form is filled as part of RACD records. The Case investigation forms are confidential and are stored by the Regional Health Directorate as part of MoHSS malaria data. Informed consent as well as the purpose of the study was explained to the patients in order for them to decide whether they wanted to participate in the study or not. Finger prick blood samples were tested by placing the blood collected on a capillary tube on the RDT designated well with buffer and after 20 minutes, the results were read out, which determined if a DBS would be collected or not. If the result was positive, the individual would be pricked again for the second time to collect blood on Whatman (3MM) filter paper and left to air-dry. Each DBS that was RDT malaria positive was labelled with a unique identifying number without the individual's identity. The DBS upon collection were stored in a -20˚C fridge at the study site and were transported to the Biomedical Research Laboratory, Windhoek, University of Namibia in a portable freezer at the end of the malaria season end of May. Samples were stored in a -20˚C until further processing. A total of 251 and 338 RDT malaria positive DBS samples were collected during the 2017 and 2021 malaria seasons respectively from February – May. Below is a map showing the study sites Sesheke and Choi circled in red.

Figure 15: Map of Health facilities in Zambezi region, study sites, Sesheke and Choi are circled in red.

Source: (Mumbengegwi et al., 2018)

3.2.2 DNA Extraction for *Plasmodium* Identity and Confirmation

The Chelex method used for DNA extraction provides a simple, cost – effective and rapid DNA extraction method from DBS (Holzschuh & Koepfli, 2022). The DBS were punched into a 2mL micro-centrifuge tube, and between each sample, the puncher was cleaned by swirling it vigorously in 70% ethanol and then in water. Then next, a blank piece of filter paper was punched three times before moving onto the next sample to prevent cross contamination. An addition of 300 µL of pre heated 20% Chelex beads (Bio Rad) in deionised water was added into each tube containing the DBS sample. The tubes were each vortexed for 30 seconds to thoroughly mix the solution, and then the tubes were placed on a heating block at 99˚C for 10 minutes to lyse the cells in the solution containing the Chelex beads. These beads bind to the metals that act as PCR inhibitors (Tani et al., 2008; Strøm et al., 2014).

The tubes were opened briefly for the first two minutes to prevent pressure build up which could potentially lead to spillage of the samples resulting in possible contamination of the other samples leading to false-positives. After the heating step, the samples were centrifuged for 2 minutes at 12000 rpm, and then the supernatant was transferred to a second set of labelled tubes, with some carry-over of the Chelex beads. The second set of tubes were centrifuged at 12000rpm for 2 minutes and transferred to a third set of labelled tubes, this time without any carry-over of Chelex beads. The extracted DNA was then stored at -20˚C (Tani et al., 2008; Strøm et al., 2014). The flow chart below summarises the Chelex extraction method.

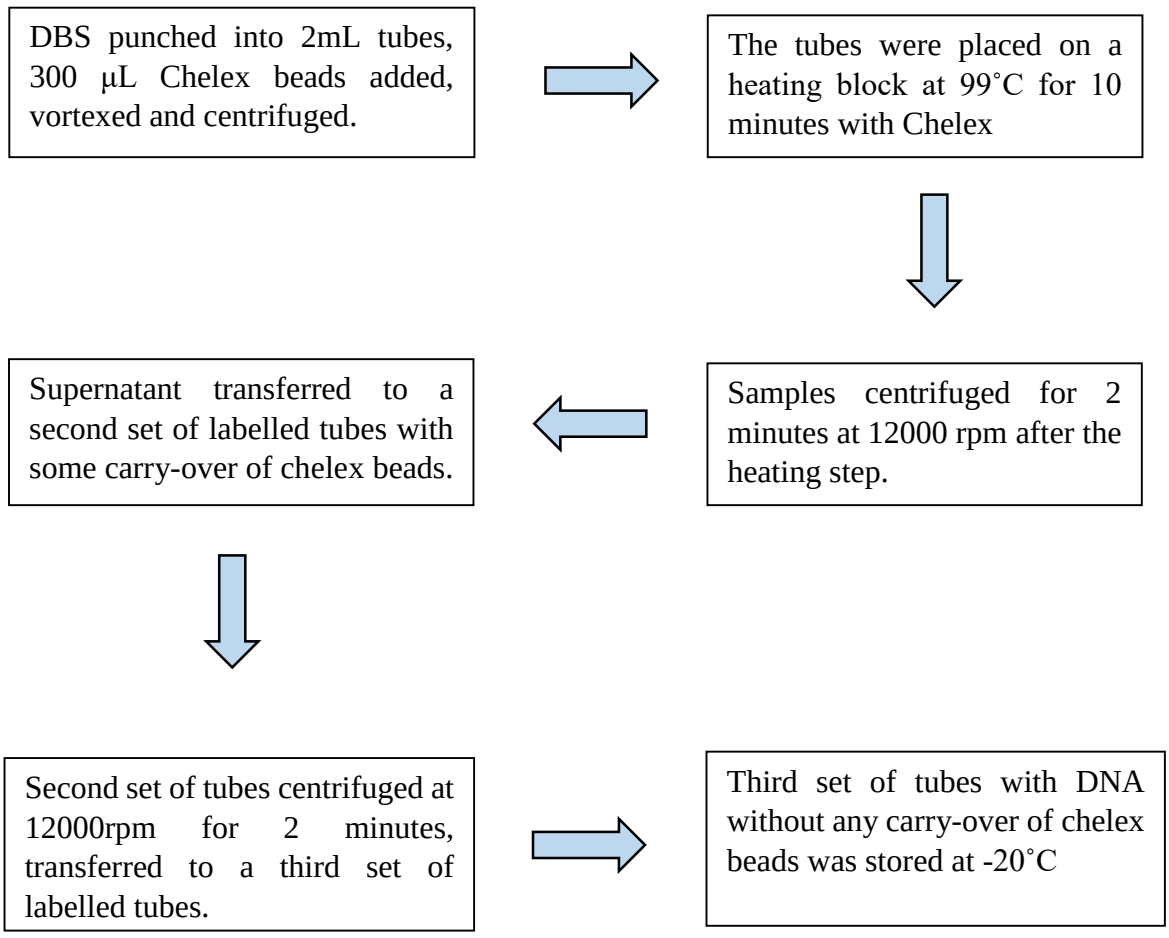


Figure 16: Flow chart showing the Chelex DNA extraction method

3.2.3 Confirmation and Identification of *Plasmodium* species by PCR

A total of 589 DBS from individuals that were malaria positive by RDT, from the Zambezi region were amplified by multiplex PCR (Padley et al., 2003). A Whatman 3MM filter paper with PCR confirmed *Plasmodium* DNA was used as a positive control for *P. falciparum*, filter paper with no blood was used as a negative extraction control, while pure master mix without a DNA template was used as a negative template control. In the PCR, a single reverse primer (5'-GTA TCT GAT CGT CTT CAC TCCC-3'), which was conserved in all four *Plasmodium* species, was used with four species-specific forward primers for: *P. falciparum* (5'-AAC AGA CGG

GTAGTC ATG ATT GAG-3'), *P. vivax* (5'-CGGCTT GGA AGT CCT TGT-3'), *P. ovale* (5'-CTG TTC TTT GCA TTC CTTATGC-3') and *P. malariae* (5'-CGT TAA GAATAA ACG CCA AGCG-3') (Padley et al, 2003). This procedure targets the conserved *18S rRNA* gene of *Plasmodium*. To each 0.2mL PCR tube with mix containing 12.5 µL of 2x quick-load master mix (New England Biolabs), 5.5 µL of nuclease free water, 1.0 µL forward primer and 1.0 µL reverse primer (at 10uM each) (Inqaba), 5 µL of extracted DNA sample was added. The cycling conditions of the PCR were: primary denaturation at 94˚C for 5 minutes followed by 35 cycles of final denaturing at 94˚C for 15 seconds, an annealing temperature at 60˚C for 30 seconds, an extension step at 68˚C for 15 seconds and a final extension step at 68˚C for 2 minutes with a holding step at 4˚C. After the multiplex PCR run, the PCR products were run on a 2.5% agarose gel which was created by mixing 3.75 grams of agarose powder and 150 mL of TE buffer. After casting the gel and placing the solidified gel in the gel tank with TE buffer, 10 uL of PCR product was added to the well created by the combs and run on 120 volts for an hour. The PCR methods were run in duplicate, and if there were any discordant results, a third run was conducted. A 100 bp ladder (Inqaba) was used, and when it ran out, a 50 bp ladder was used. The presence of the *Plasmodium* species is associated with different band sizes (*P. falciparum* – 276 bp; *P. vivax* – 300 bp; *P. ovale* – 375 bp; *P. malaria* – 412 bp). Below is a table summarising the primer names and sequences.

Table 2: Summary of the primer names and sequences used in the reaction mix

Primer Name	**Primer Sequence**
Forward Primer (*P. falciparum*)	5'-AACAGACGGGTAGTCATGATTGAG-3'
Forward Primer (*P. vivax*)	5'-CGGCTT GGA AGT CCT TGT-3'
Forward Primer (*P. ovale*)	5'-CTG TTC TTT GCA TTC CTTATGC-3'
Forward Primer (*P. malariae*)	5'-CGT TAA GAATAA ACG CCA AGCG-3'
Reverse Primer	5'-GTATCTGATCGTCTTCACTCCC-3'

3.2.4 DNA Extraction Using the QIAamp DNA extraction Kit

The QIAamp DNA extraction kit (Qiagen) was used to extract DNA in comparison to the Chelex method. DNA extracted using the Chelex method has been reported to contain potential contaminants that can lead to inaccurate qPCR. A total of 223 DBS that were PCR positive were punched using a single hole puncher into their corresponding 2 mL micro centrifuge labelled tubes with 180 µL of Buffer ATL added to the tubes. The tubes were incubated at 85°C for 10 min. After 10 minutes the tubes were briefly centrifuged to remove drops from inside the lid. Then 20 µL of proteinase K stock solution was added to the tube, mixed by vortexing and incubated at 56°C for 1 hour. After the 1h incubation period, the tubes were briefly centrifuged to remove drops from inside the lid and 200 µL of Buffer AL was added to the sample, mixed thoroughly by vortexing and incubated at 70°C for 10 minutes. After the 10 minutes, the tubes were briefly centrifuged to remove drops from inside the lid.

A total of 200 µL ethanol (100%) was added to the sample, mixed thoroughly by vortexing and briefly centrifuged to remove drops from inside the lid. The mixture

was then carefully applied to a QIAamp Mini spin column (in a 2 mL collection tube) without wetting the rim and was centrifuged at 8000 rpm for 1 minute. Then the closed QIAamp Mini spin column was placed in a clean 2 mL collection tube, and the tube containing the filtrate was discarded. A total of 500 µL Buffer AW1 was then added to the QIAamp Mini spin column without wetting the rim, the caps were closed and centrifuged at 8000 rpm for 1 minute. The QIAamp Mini spin column was placed back in the same collection tube after the filtrate was discarded, then 500 µL Buffer AW2 was added without wetting the rim.

The tubes were centrifuged at 8000 rpm for 1 minute, then the filtrate was discarded and the QIAamp Mini spin column was placed back in the same collection tube. The tubes were closed and centrifuged at full speed for 10 minutes to dry the membrane. The QIAamp Mini spin column was placed in a new 2 mL collection tube and the old collection tube was discarded with the filtrate. A total of 100 µL of Buffer AE was added to the QIAamp Mini spin column and was centrifuged at full speed for 1 minute at 8000 rpm to elute the DNA, the eluted DNA was placed in a clean 0.5 mL tube and stored at -20˚C until further use. Multiplex PCR was conducted again on the QIAamp extracted samples the same as in heading 3.2.3 to confirm the positive bands with the bands extracted by Chelex. The PCR products were run on a 2.5% agarose gel which was created by mixing 3.75 grams of agarose powder and 150 mL of TE buffer. After casting the gel and placing the solidified gel in the gel tank with TE buffer, 10 uL of PCR product was added to the well created by the combs and run on 120 volts for an hour. The flow chart below summarises the steps used for DNA extraction using the QIAamp DNA extraction kit.

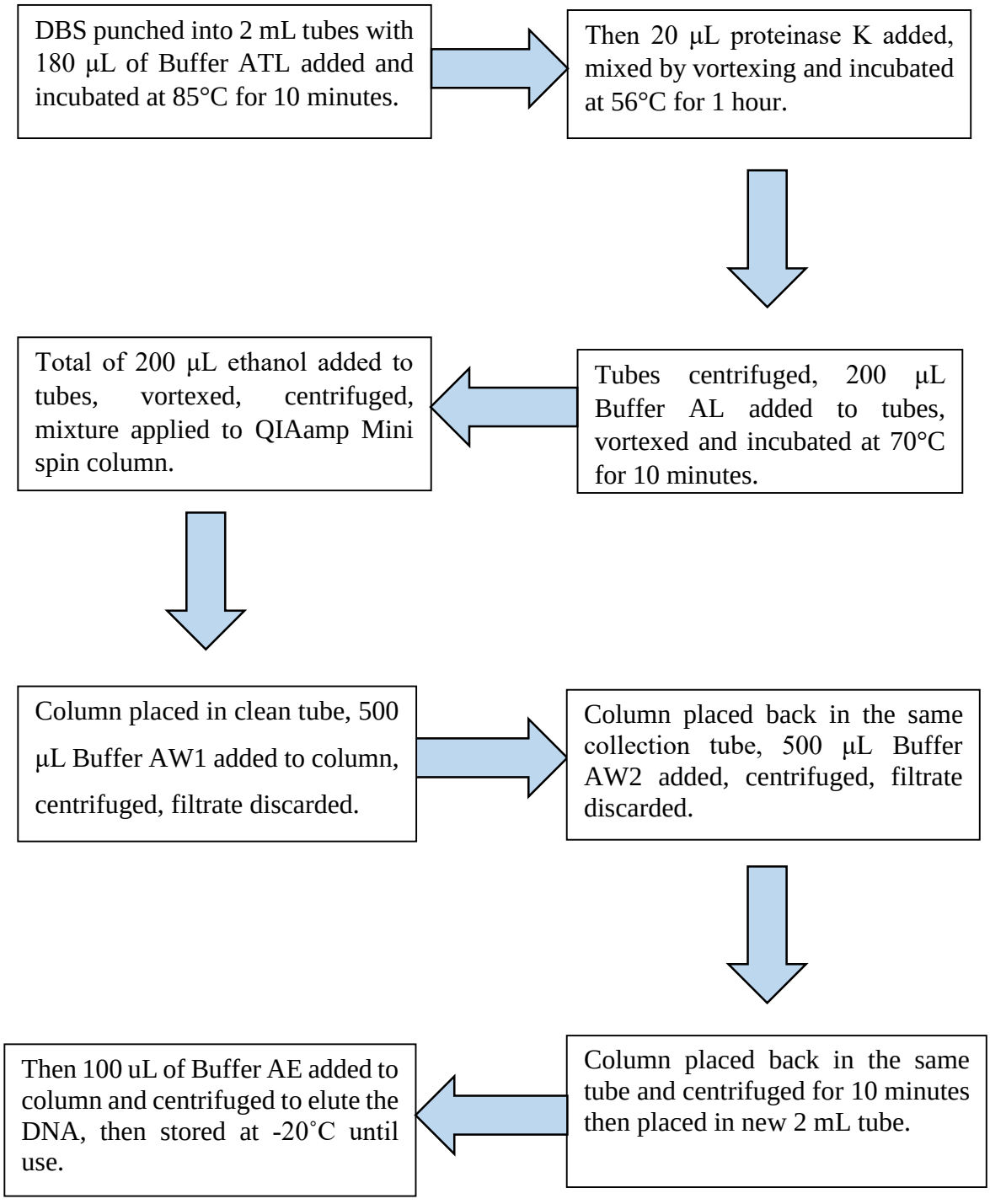


Figure 17: Flow chart of the DNA extraction method using the QIAamp DNA extraction kit

3.2.5 Determination of Parasite Density targeting the var gene Acidic Terminal Sequence (varATS) using qPCR

The extracted DNA was used for qPCR targeting the varATS gene of *Plasmodium falciparum* (Hofmann et al., 2015) using the Probe sequence (6-FAM-TRTTCCATAAATGGT-NFQ-MGB), the forward primer (5'-CCCATACACAACCAAYTGGA-3'), and reverse primer (5'-TTCGCACATATCTCTATGTCTATCT -3'). A Whatman 3MM filter paper with PCR confirmed *Plasmodium* DNA was used as a positive control for *P. falciparum,* filter paper with no blood was used as a negative extraction control, while pure master mix without a DNA template was used as a negative template control. A standard curve was generated using known parasite density of a 3D7 strain. Ten-fold serial dilutions of the extracted DNA template were prepared in the following order: 10^4, 10^3, 10^2,10^1,10^0,10^{-1}. The threshold cycles were plotted versus the dilution factor and were fitted to a straight line. Each reaction tube with 5 µL of DNA template contained 5 µL of nuclease free water, 10 µL of 2x SensiFast master mix (Meridian Bioscience), 2 µL of varATS forward primer at 10uM (Inqaba), 2 µL of varATS reverse primer at 10uM (Inqaba), and 1 µL of varATS probe at 10uM (Inqaba), all these volumes added up to a total reaction volume of 25 µL per tube. Below is a table summarising the primers and sequences.

Table 3: Summary of the primers for *Plasmodium falciparum* and sequences used in the reaction mix

Primer Name	**Primer Sequence**
Forward Primer	5'-CCCATACACAACCAAYTGGA-3'
Reverse Primer	5'- TTCGCACATATCTCTATGTCTATCT -3'
Probe Sequence	6-FAM-TRTTCCATAAATGGT-NFQ-MGB

Parasite densities were calculated and used as a proxy for transmission intensity, the Ct values obtained during qPCR reflect the amount of concentration of the target gene present, the lower the Ct value, the higher the concentration of the target gene and the higher the Ct value, the lower the concentration of the target gene (Guiguemde et al., 2020). After extraction with the QIAamp DNA extraction kit and qPCR, the efficiency of the assay was 103%, and the Ct values, gradient and y- intercept obtained from the run were used to calculate parasitaemia based on this formula:

Ct= m (log quantity) = b

Where Ct: is the Cycle threshold value

m: is the gradient

b: is the y intercept

making "quantity" the subject of the formula:

Quantity = $10^{((Ct-b)/m)}$ (Bio Rad, 2006; Gruenberg et al., 2020)

Below is a table showing the parasite case definitions based on parasite density ranges and where used to categorise the results obtained from this study after quantifying the parasite densities.

Table 4: Parasitaemia case definitions: low, moderate, high, hyperparasitaemia based on the parasite density ranges

Source: (Chipwaza & Sumaye, 2020)

Parasitaemia Case Definitions	**Parasite Density Ranges**
Low	<1000 parasites/µL
Moderate	1000-4999 parasites/µL
High	5000-99999 parasites/µL
Hyperparasitaemia	≥100000 parasites/µL

3.2.6 RNA Extraction for Gametocyte mRNA

The Quick-RNA™ Mini-prep Plus Kit (Zymo Research) was used to extract mRNA (Kosasih et al., 2021). To each 2 mL tube with DBS, 300 µL of DNA/RNA Shield (1X) was added with 15 µL of Proteinase K and 30 µL of PK Digestion Buffer. The tubes were briefly vortexed and incubated at room temperature for 60 minutes. After incubation, the samples were vortexed and centrifuged at maximum speed (12000g) for 2 minutes. Then 300 µL RNA Lysis Buffer was added to the same tube. The lysed sample was transferred into a Spin-Away Filter in a collection tube and centrifuged to remove the majority of genomic DNA. One volume of ethanol (100%) was added to the flow-through and mixed.

The sample was then transferred into a Zymo-Spin™ IIICG Column1 in a collection tube and centrifuged. The flow through was discarded and 400 µL RNA Wash Buffer was added to the column and centrifuged. The flow through was discarded. In a nuclease-free tube, 5 µL DNase I and 75 µL DNA Digestion Buffer were mixed together and added directly onto the column matrix. The column matrix was incubated at room temperature (20-30°C) for 15 minutes. Then 400 µL of RNA Prep Buffer was added to the column and centrifuged.

The flow-through was discarded and 700 µL of RNA Wash Buffer was added to the column and centrifuged and the flow-through discarded. A total of 400 µL of RNA Wash Buffer was added to the column and centrifuged for 1 minute to ensure complete removal of the wash buffer. Then the column was transferred into a nuclease-free tube and 100 µL DNase/RNase-Free Water was added directly to the column matrix and centrifuged. The eluted RNA was then stored in a -80˚C freezer. The flow chart below shows the summarised RNA extraction steps using the RNA extraction kit from Zymogen.

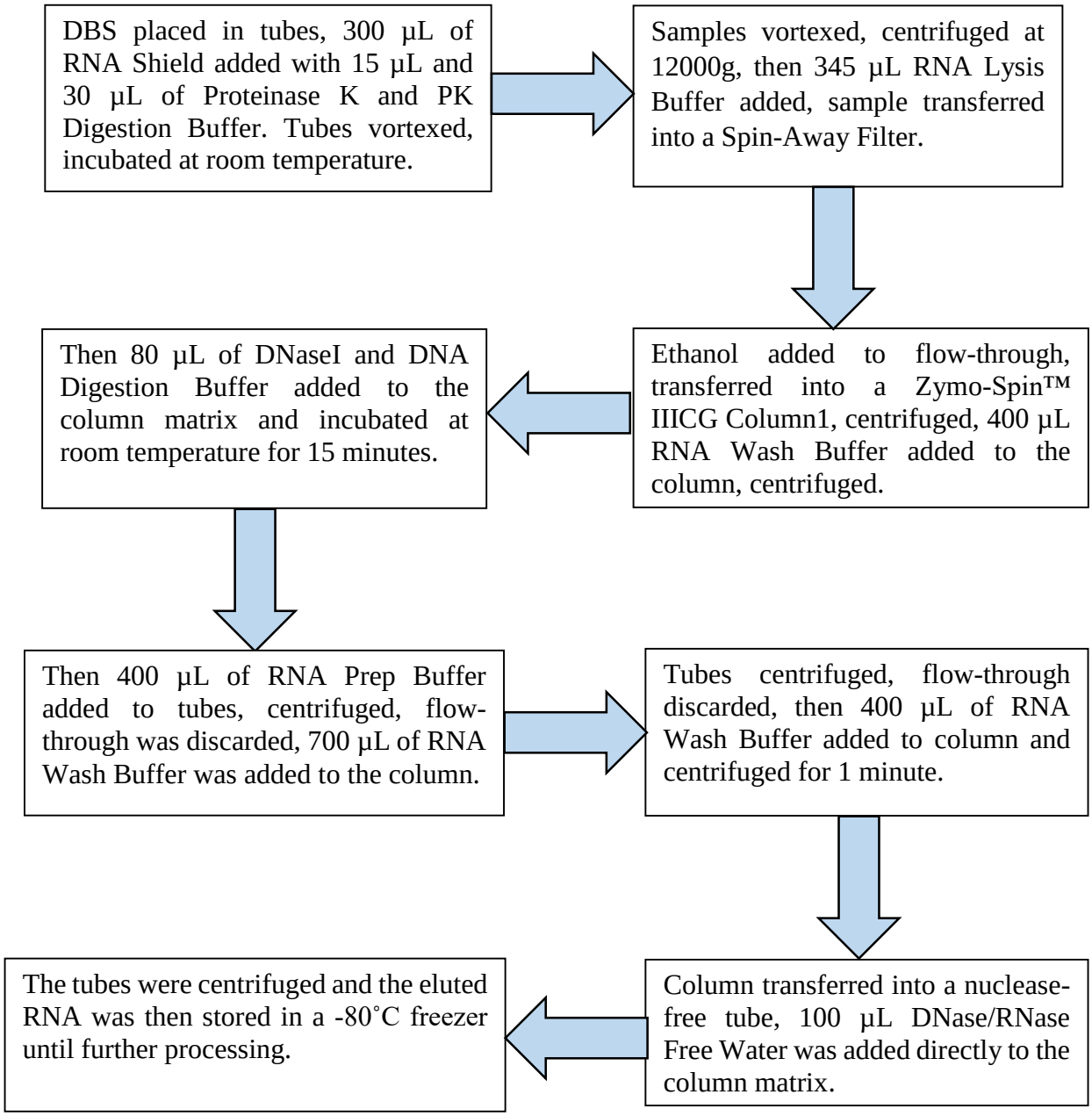


Figure 18: Flow chart showing the summarised RNA extraction method

3.2.7 Detection and Quantification of Gametocytes using RTqPCR

For the *Pfs25* gametocyte assay, the forward primer that was used was (5'-CCATGTGGAGATTTTTCCAAATGTA-3'), the reverse primer: (5'-CATTTACCGTTACCACAAGTTACATTC-3'), and the probe (6FAM-CCGTTTCATACGCTTGTAA-MGB) (Schneider et al., 2015). Filter paper with dried blood containing gametocytes were used as a positive extraction control for *P. falciparum*, filter paper with no blood was used as a negative extraction control and a minus reverse transcriptase control was used to test for any contaminating DNA such as genomic DNA or PCR product from previous run. A standard curve was generated using known gametocyte density of a NF54 strain. Ten-fold serial dilutions of the extracted DNA template were prepared in the following order: 10^4, 10^3, 10^2,10^1,10^0,10^{-1}. The threshold cycles were plotted versus the dilution factor and were fitted to a straight line. The amplification of the *Pfs25* gene allowed the detection of gametocytes and the fluorescence emission determined the cycle threshold (Ct) that reflected the initial amount of cDNA. The total reaction mixture of 20 µL comprised of 3 µL of template RNA, 10 µL of Luna Universal Probe One-Step RTqPCR (New England Bio labs), 1 µL of Luna Warm-Smart RT Enzyme Mix (New England Bio labs), 0.8 µL of forward and reverse primer and 0.4 µL probe (10µM forward and reverse primers, and 5µM MGB probe) (Inqaba), and 4 µL of nuclease-free water. The cycling conditions were as follows: 55°C for 10 minutes for synthesis of the cDNA and then 95°C for 1 minute, followed by 45 cycles of 95°C, 10 s; and 60°C, 1 minute. Below is a table summarising the primers and sequences.

Table 5: Primer sequences for RTqPCR of samples for *Plasmodium falciparum* female gametocytes (*Pfs25* gene)

Primer Name	**Primer Sequence**
Forward Primer	5'-CCATGTGGAGATTTTTCCAAATGTA-3'
Reverse Primer	5'-CATTTACCGTTACCACAAGTTACATTC-3'
Probe Sequence	6FAM-CCGTTTCATACGCTTGTAA-MGB

The use of RTqPCR assays for detection of late gametocyte stages can reveal the high transmission capacity of the human malaria parasite, *Plasmodium falciparum*. A total of 223 samples that were confirmed as *P. falciparum* by PCR were used for RNA extraction and RTqPCR was performed on these samples. After the RTqPCR run, the efficiency of the assay was 96%, and the Ct values, gradient and y- intercept obtained from the run were used to calculate gametocytaemia based on this formula:

Ct= m (log quantity) = b

Where Ct: is the Cycle threshold value

m: is the gradient

b: is the y intercept

making "quantity" the subject of the formula:

Quantity = $10^{((Ct-b)/m)}$ (Bio Rad, 2006)

3.3 Data Analysis

A normality test was conducted on data from parasitaemia and gametocytaemia following quantification using qPCR and RTqPCR. A p-value below 0.05 showed that the data was not normally distributed, therefore, the non-parametric Mann–Whitney

test was used to compare samples with a non-normal distribution for parasitaemia from 2017 and 2021 as well as gametocytaemia from both seasons. Spearman's correlation coefficient (*rho*) was used to determine the correlation between parasite density and *Pfs25* gametocyte carriage. The medians for 2021 using the median test was also used to analyse if there were any significant differences in gametocytaemia based on parasite case definitions. All analyses were done with the SPSS statistical software for Windows, Version 28.

3.4 Research Ethics

Ethical approval for this study was obtained from the Decentralised Ethics Committee (SOS-0029) (Appendix 1, page 132) before samples were collected. The study and recruitment process were explained to the participants in their own language and written informed consent and assent of the participants or their parents/guardians/representatives was received before testing and sample collection. The names of the participants were kept anonymous by assigning a number to each participant for those that volunteered. Samples were collected and handled by trained nurses and there was negligible risk to participants as finger prick for RDT testing is a standard of care, the only risk would be mild pain and discomfort during blood collection from the finger prick. Sterile techniques were enforced at all times during the study.

Chapter 4. Results

4.1 Confirmation and Identification of *Plasmodium* species by PCR

A total of 589 DBS from RDT positive malaria cases from the outbreak seasons 2017 and 2021 were analysed as use cases for molecular tools in surveillance of malaria at low transmission settings. The flow chart in Figure 19 below summarizes the results of the samples analysed and the parameters that were assessed. For the 2017 outbreak (February – May), DNA was extracted from a total of 251 RDT positive DBS whilst for the 2021 outbreak (February - May 2021), 338 DBS samples were used. Only 71/251 (28%) *P. falciparum* positive samples by PCR were confirmed for the year 2017 and 152/338 (45%) for the year 2021.

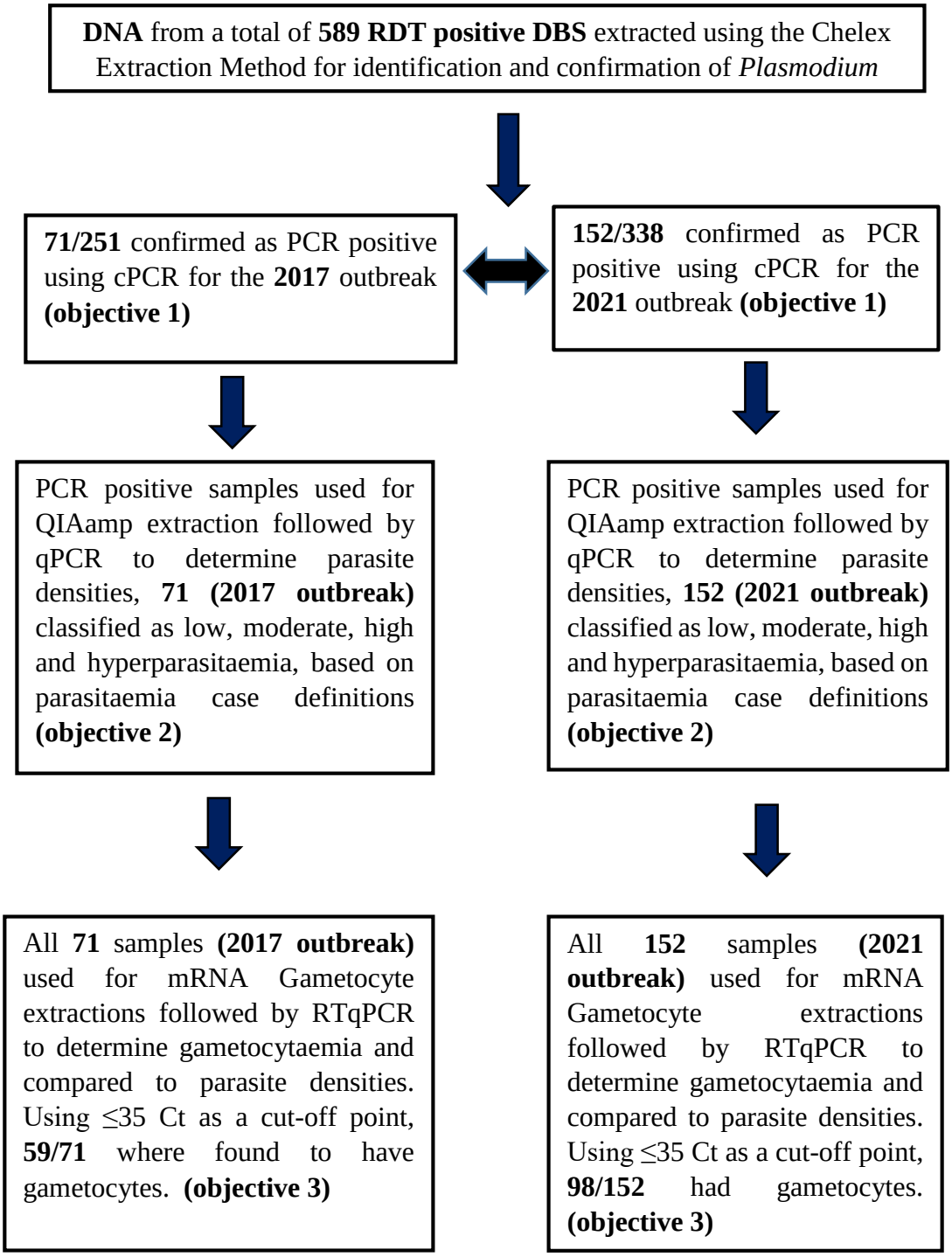


Figure 19: Work flow of sample analysis

Figures 20 below show representative gel results after multiplex PCR for confirmation and speciation of blood samples from individuals with malaria positive RDTs. This means the RDTs for these samples were false-positive for *P. falciparum*. All samples positive by PCR for both outbreaks were confirmed to be *P. falciparum*, none were *P. vivax*, *P. ovale* or *P. malariae*.

Figure 20 shows the gel results for the 2021 samples following conventional PCR. The numbers in wells 1-22 with a band correspond to the positive extraction control (PEC) as well as the positive template control (PTC), which identifies them as *Plasmodium falciparum*, the lanes without a band are PCR negative, no other *Plasmodium* species were found.

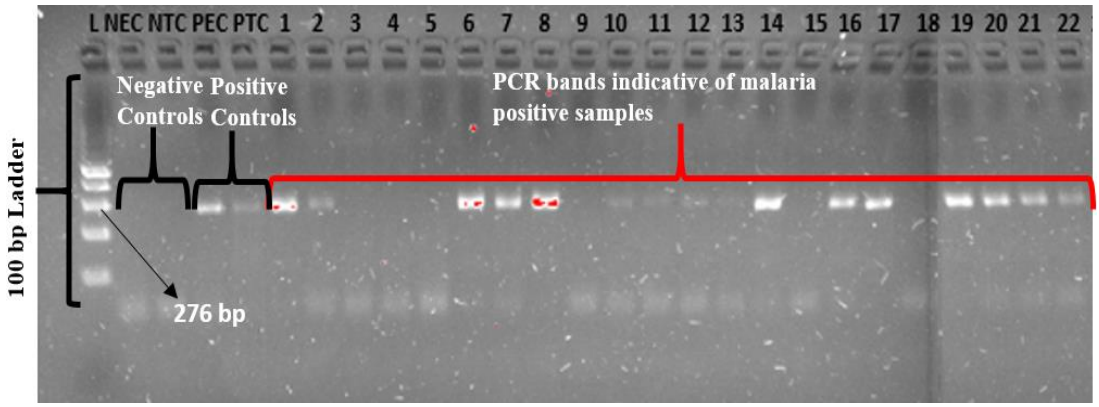


Figure 20: Representative gel showing presence of P. *falciparum* in 2021 outbreak samples using conventional PCR as compared to the 100 bp ladder and controls, NEC (negative extraction control), NTC (negative template control), PEC (positive extraction control for *P. falciparum*, 276 bp) and PTC (positive template control for *P. falciparum, 276 bp).*

4.2 Determination of Parasite Density targeting the var gene Acidic Terminal Sequence (varATS) using qPCR

All PCR positive samples in Section 4.1 were extracted using the Chelex method as described in Section 3.2.2 and were used for qPCR. The extractions did not yield accurate results due to the impurities that were present with the DNA in solution as the derived Ct values were high (>35) or could not be derived. Therefore, the QIAamp DNA extraction kits were used to re extract DNA for use in qPCR as they have been reported to yield pure DNA and is effective at removing impurities (Holzschuh & Koepfli, 2022). A typical standard qPCR amplification curve has three distinct phases: (1) a baseline that gradually transitions into (2) an exponential region, followed by (3) a plateau, which indicates that amplification is reducing (Hsu, Sherina & McCall, 2020), however this was not observed in samples that were extracted using the Chelex method, but was observed in samples that were extracted using the QIAamp kit. Figure 21 below shows results of the qPCR with DNA extracted using the Chelex method (left) and the QIAamp kit (right).

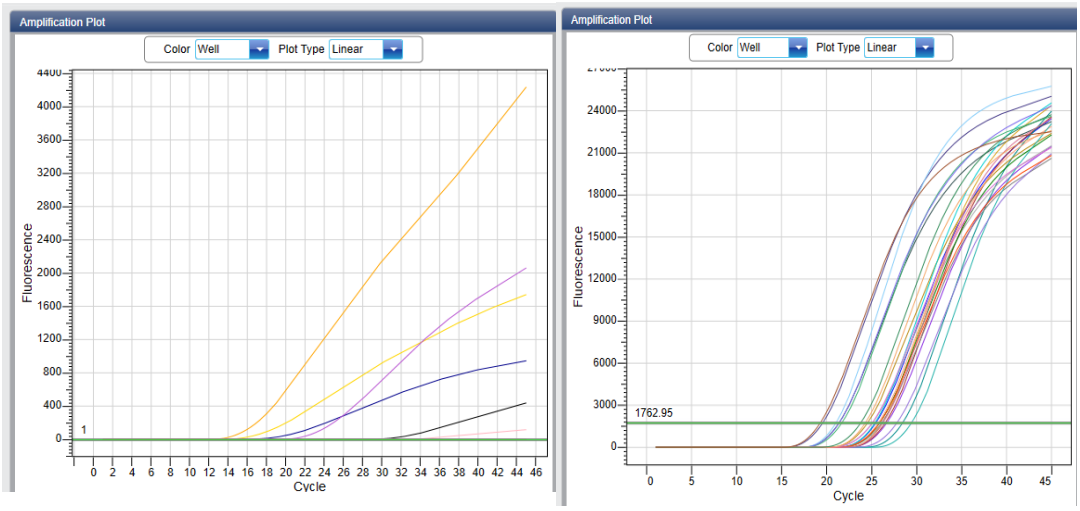


Figure 21: Quantitative Polymerase Chain Reaction curves obtained using samples extracted by Chelex extraction (left) and samples extracted with the a QIAamp kit (right). Minimum cycles (n=45)

The formula: Quantity = $10^{((Ct-b)/m)}$ was used to calculate the parasite densities for all 223 samples and were categorised as either low, moderate, high or hyperparasitaemia and were categorised according to table 4. Figure 22 shows the parasite densities for 2017 and 2021 categorised based on the parasite density ranges.

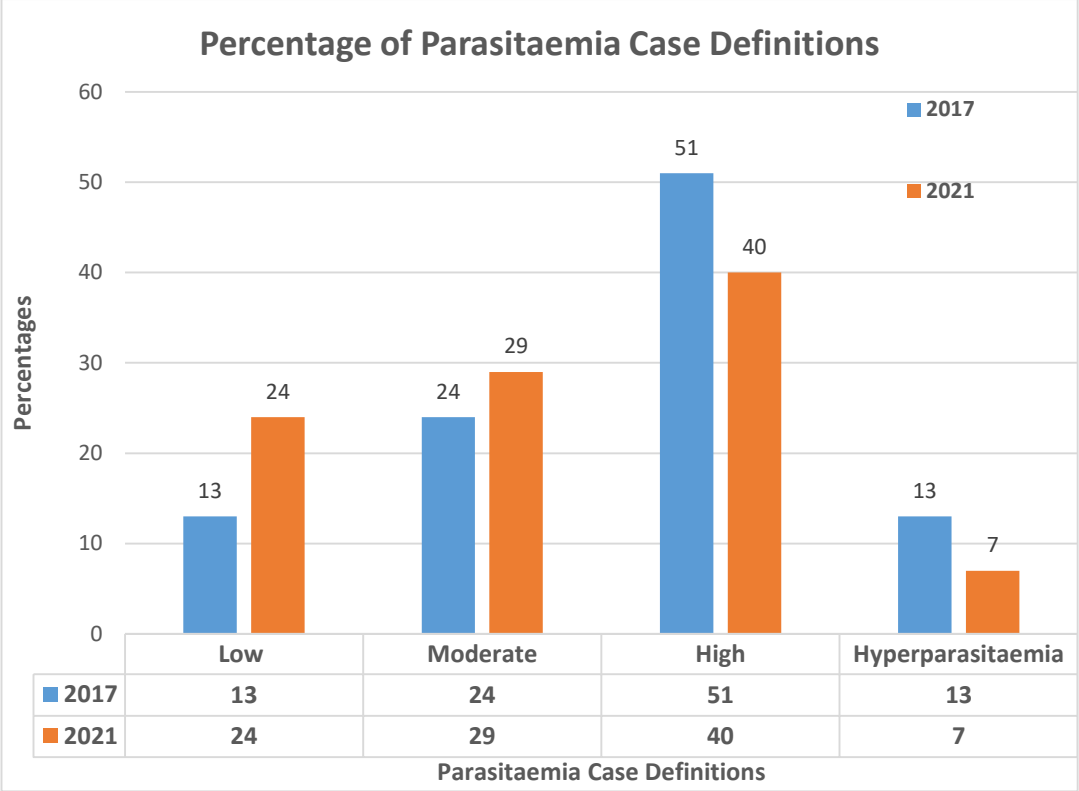


Figure 22: Classification of parasitaemia case definitions of individuals with PCR positive confirmed cases (low, moderate, high and hyperparasitaemia) for both 2017 and 2021 expressed as percentages based on parasite density.

The parasite densities from samples collected from both outbreaks ranged from low to hyperparasitaemia with a larger proportion of samples exhibiting high parasitaemia followed by moderate parasitaemia (40% and 29% for 2021 and 51% and 24% for 2017). The trend for low and hyperparasitaemia for 2021 was different with 24% of samples showing low parasitaemia and only 7% showing hyperparasitaemia. The 2017 outbreak showed similar proportions of low parasitaemia and hyperparasitaemia at 13%. The data was not normally distributed, as the normality test for both outbreaks had a p-value below 0.05 (2017 p=0.001 and 2021 p=0.001). The Mann-Whitney test was used to compare means. The Mann-Whitney test ($p = 0.008$), confirmed that there is a significant difference between the mean parasitaemia for both years.

Quantification of parasitaemia from the 2017 outbreak samples showed that there was a mean parasite density of 46088 p/µL, which was significantly higher than the mean parasite density of samples from the 2021 outbreak season (35211 p/µL) as confirmed by Mann-Whitney, the significant difference.

4.3 Detection and Quantification of Gametocytes using RTqPCR

The formula: Quantity = $10^{((Ct-b)/m)}$ was used to determine gametocytaemia using the Ct values, y-intercept and gradient from the reaction (Bio Rad, 2006). A Ct value of 35 and below was used as a cut-off point to determine presence of gametocytes (Guiguemde et al., 2020). The data was not normally distributed, as the normality test for both outbreaks had a p-value below 0.05 (2017 p=0.001 and 2021 p=0.001). The Mann-Whitney test was used to compare gametocytaemia means. There was a significant difference in calculated gametocytaemia mean between 2017 and 2021 (Mann-Whitney test, p = 0.003) with 2021 samples showing a significantly higher gametocytaemia mean (892 g/µL) as compared to 2017 (116 g/µL). Low parasitaemia cases for both outbreaks were shown to also carry gametocytes. The table below shows the detected gametocytes based on the parasite case definitions and density ranges.

Table 6: Detected gametocytes based on the parasite case definitions

Parasitaemia Case Definitions	**Parasite Density Ranges**	**Samples with Present Gametocytes (2017)**	**Samples with Present Gametocytes (2021)**
Low	<1000 parasites/µL	5/59 (8%)	14/98 (14%)
Moderate	1000-4999 parasites/µL	10/59 (17%)	27/98 (28%)
High	5000-99999 parasites/µL	35/59 (59%)	47/98 (48%)
Hyperparasitaemia	≥100000 parasites/µL	9/59 (15%)	10/98 (10%)

The correlation for the mean parasitaemia values for each parasite case definition (low, moderate, high and hyperparasitaemia) and gametocytaemia were determined. For 2021, the *rho* value was 1 and for 2017 was 0.6 which show a strong correlation between parasitaemia and gametocyte carriage for 2021 and moderate correlation for 2017, meaning the higher the parasite density, the likely the presence of infectious gametocytes that can be taken up by mosquitoes for onward transmission. Hence parasite density with present gametocytes increases risk of transmission of malaria parasites.

Figure 23 and 24 below show box plots for both 2021 and 2017 gametocytaemia based on parasite case definitions, samples with high parasitaemia were more likely to carry gametocytes for samples analysed from both 2017 and 2021 samples.

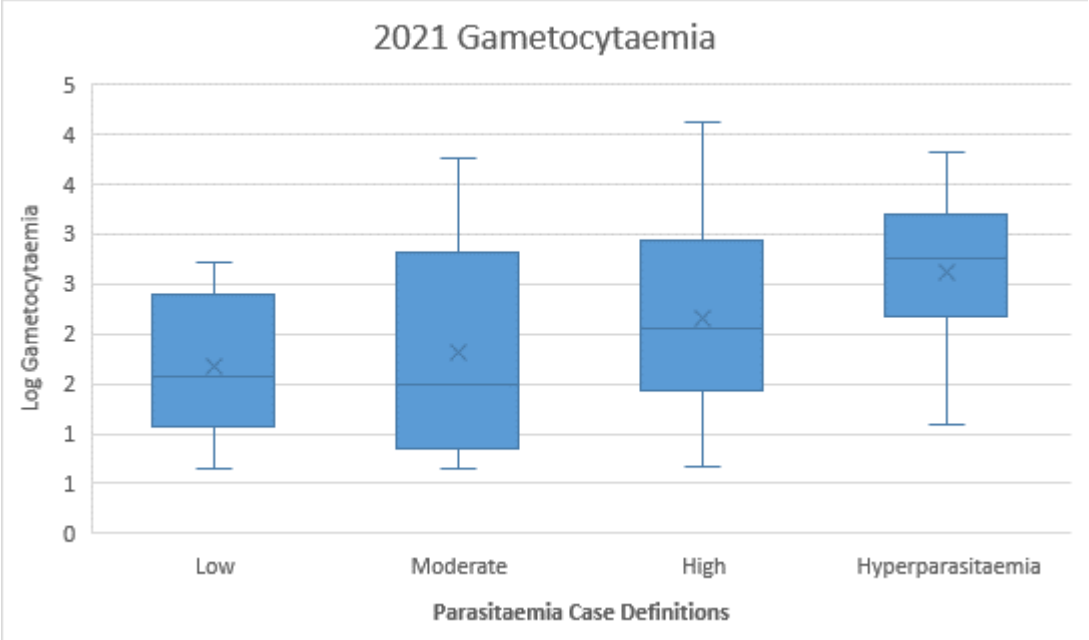


Figure 23: Box plot showing the distribution patterns for gametocytaemia based on classification of parasitaemia case definitions for samples from the 2021 malaria outbreak Season.

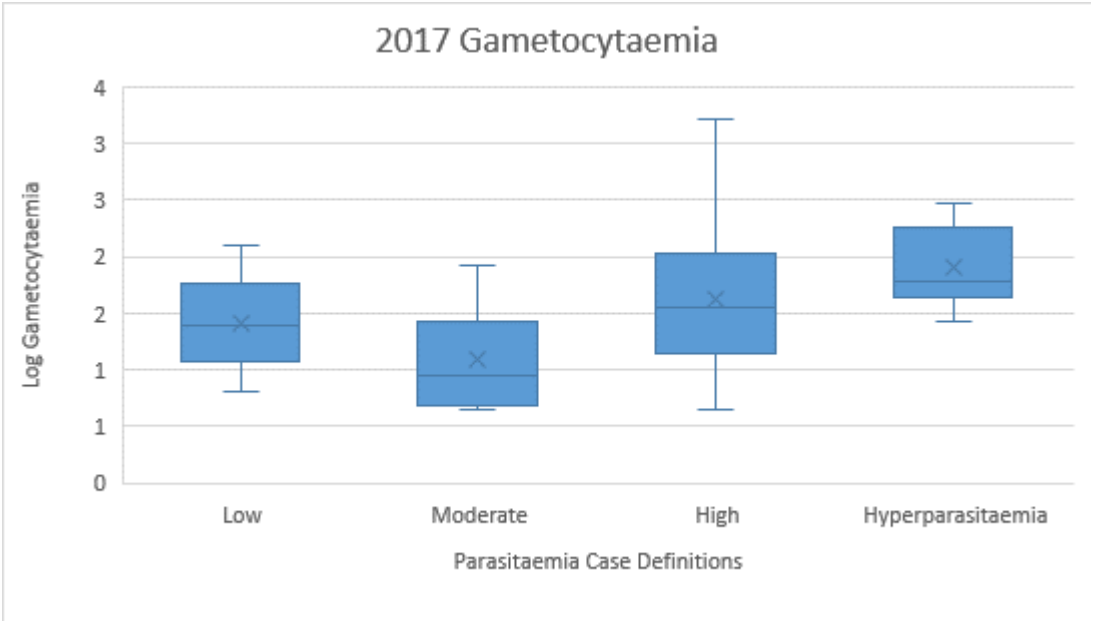


Figure 24: Box plot showing the distribution patterns for gametocytaemia based on classification of parasitaemia case definitions for samples from the 2017 malaria outbreak Season.

The box plot trends which compared the median gametocytaemia for both 2021 and 2017, showed that the low parasitaemia for 2021 had a higher median of gametocytes than the moderate parasitaemia, and for 2017, the low parasitaemia also had a higher median of gametocytaemia than the moderate parasitaemia. Interestingly, the Median Test analysis in SPSS showed that there was no significant difference between the medians of gametocytaemia for low and moderate parasitaemia for 2021 ($p=0.910$) meaning low parasitaemia infections had similar levels of gametocytes as moderate parasitaemia. For 2017, the sample size was not large enough to provide enough valid cases to perform the Median Test for Gametocytaemia 2017 * Parasitaemia 2017, hence this relationship was not analysed statistically.

Chapter 5. Discussion

Due to the malaria outbreaks that have occurred in Northern Namibia, the progress towards malaria elimination has stalled in the last five years. RDTs used for malaria case management and surveillance have shown limited sensitivity in either not detecting all cases at low transmission settings such as Zambezi region or in showing false-positive results due to HRP-2 persistence. False-negative results lead to undetected and untreated infections that can still perpetuate transmission while false-positives lead to over treatment, waste of resources and can possibly drive development of drug resistance in parasites (Tedla, 2019).

In this study, samples from the 2017 outbreak showed that 28% were PCR positive for *Plasmodium falciparum* malaria while samples for the 2021 outbreak had a higher concordance of 45% samples being both RDT and PCR positive for *Plasmodium falciparum*. No other *Plasmodium* species were found in all 589 samples. On average, 38% of the 589 samples from the two malaria outbreak seasons 2017 and 2021 were confirmed by PCR as being malaria positive with 62%, (almost 2 out of every 3 samples) being false-positives. Haindongo (2016) showed that PCR detected 33% of malaria infections compared to RDTs in Outapi and Oshikuku (both sites in Namibia), highlighting the high false positivity (Haindongo, 2016). The high false positivity observed in this study is consistent with other studies (Hosch et al., 2022) and can be attributed to the persistence of the HRP-2 antigenemia after successful treatment. Indeed, it has been observed that RDT detectable HRP-2 antigenemia can last up to 28 days post successful treatment, thus increasing chances of false positives (Mayxay et al., 2001). However, since this study contributes to malaria surveillance, it was important to further test the samples because of the possibilities of either antigen persistence, re-infections or delayed parasite clearance/resistance in previously tested

patients and cross reactivity with other diseases such as Trypanosomiasis and Rheumatoid factor (Gatton et al., 2018). According to MoHSS guidelines, patients with history of infection are not treated, but samples are collected for surveillance purposes due to other possible reasons for RDT positive results. If the samples were both RDT and PCR positive, it could either be a new infection or delayed parasite clearance due to ineffective drug against the parasite. The high false positivity rate limits RDT usefulness in the management of fever cases (Michael, Orimadegun & Falade, 2021) as they could lead to overtreatment, waste of resources and possible risk of drug resistance (Fitri et al., 2022). Therefore, the high proportion of false-positive RDTs from this study of 62% may be due to *P. falciparum* histidine rich protein 2 (pfhrp2) antigen persistence after recent malaria treatment. This may have been because the study was conducted in a malaria transmission hotspot and repeat visits during RACD may have led to oversampling of persons who had recently recovered from malaria.

RDTs can also produce false positive result in patients with trypanosomiasis and rheumatoid factor. Since rheumatoid factor seroprevalence is associated with viral, parasitic and chronic bacterial infections, false positive rates may be a concern in malaria endemic countries with high prevalence of hepatitis B and C (Gatton et al., 2018). In the elimination era, awareness of the possibility of false-positive malaria RDTs in those with infections with neglected tropical diseases such as Human African Trypanosomiasis, Leishmaniasis and schistosomiasis should be considered as important for tropical regions where there is no quality-assured microscopy available to confirm malaria diagnosis. The use of RDTs may assist clinicians in initiation of early therapy. However, quality-assured examination by microscopy should be performed (Unterborn et al., 2022). Therefore, these highlight the issues of RDTs and

this confirms the first study hypothesis that RDTs do not give an accurate representation of malaria cases.

This study traced the index cases from the clinic only if they tested positive back to their homesteads. It did not sample RDT negative cases which may have had false-negatives due to low sensitivity and might have missed patients with low density infections that could have potential secondary cases back home which poses a risk for onward transmission. Sampling of RDT negative samples will be important in future studies (as infections classified as low parasitaemia infections in this study were shown to carry gametocytes). This is an important population to identify and monitor at low transmission settings as they could be the reservoir for residual transmission. Several other studies have also shown that low parasitaemia infections carry gametocytes (Chourasia et al., 2017). This group of individuals is of importance in low transmission setting where sub-patent infections make up the majority of the cases. The missed sub-patent infections could sustain malaria transmission which can delay and compromise elimination efforts (Tambo et al., 2018; Ouma et al., 2022). The high number of these individuals in many endemic settings makes sub-microscopic gametocyte carriers potentially significant contributors to malaria transmission.

Lower transmission settings like Zambezi region present unique challenges for surveillance given the small number of infected individuals and increased heterogeneity of transmission (Jacobson et al., 2019). Asymptomatic individuals are mostly not identified through passive case detection at clinics making it difficult to eliminate malaria. Tracing index cases can identify and lead to treat any secondary cases living in close proximity to or with the index case. These hotspots may be single or groups of households which experience higher levels of transmission relative to

others in the community (Smith, Auala, Tambo, et al., 2017). A previous study in Namibia found that RACD using RDTs only identified up to one-third of infections detected by more sensitive molecular methods (Smith, Auala, Tambo, et al., 2017). Smith et al., (2017) also reported that secondary infections cluster around index cases in Namibia, however, due to low parasite densities, the low diagnostic sensitivity of RDTs critically limit the use of RACD to identify and treat all infections (Smith, Auala, Tambo, et al., 2017). RACD targets asymptomatic infections and hotspots of transmission, but available point-of-care diagnostic tests (RDTs) are insufficiently sensitive and miss most infections. Targeted drug administration and vector control to smaller populations of people who are at higher risk of malaria, known as rfMDA and Reactive focal indoor residual spraying with pirimiphos-methyl builds on the existing RACD infrastructure and were found to effectively decrease the incidence and prevalence of malaria (Hsiang, Ntuku, et al., 2020).

Disease surveillance systems are important for effective disease intervention and control by monitoring disease prevalence. Understanding the contributing reasons to outbreak occurrence in low transmission settings enables to provide early case management, identify factors that maintain the disease, design and improve more effective prevention and control measures to facilitate malaria elimination and predict how to handle possible future outbreaks. Therefore, there is need for more sensitive and specific tools for malaria surveillance especially for low parasitaemia infections at low transmission settings (McMorrow, Aidoo & Kachur, 2016). Identification of parasite species is also important as when transmission of one species of Plasmodia decreases, the presence of other species increases (Akala et al., 2021). This is particularly the case where there is importation of malaria through movement of high-risk groups. A previous study showed the presence of other species such as *P. vivax*,

P malariae and *P ovalae* in Zambezi region mostly due to importation (Dausab, 2018). Haiyambo et al., (2019) also confirmed presence of non-falciparum infections in Namibia fuelled by importation, as participants that where recruited had travel history to Angola, and therefore concluded that the agenda for malaria elimination should not only focus on *P. falciparum* infections but done in parallel with non-falciparum infections as well (Haiyambo et al., 2019). However, the current study showed that all cases sampled in the two malaria outbreak seasons in 2017 and 2021 were driven by *P. falciparum* only which is historically reported as the most prevalent Plasmodia species in the region (Belete, 2020). These results were not too surprising for 2021 outbreak as it occurred during the COVID-19 pandemic where there was low population movement and lower risk of importation of other Plasmodia species. For 2017 it was expected to find non-falciparum infections, because other studies by Haiyambo et al., (2019) and Dausab (2019) showed the presence of other species due to importation (Dausab, 2018; Haiyambo et al., 2019). However, in this current study only *P. falciparum* where found and this could be due to sampling in one area.

Currently recommended tools by the WHO such as microscopy and RDTs have limitations when it comes to sub-microscopic infections, hence the need for surveillance with more sensitive and specific tools such as molecular tools that can also be used for quality assurance (QA) for these WHO recommended tools (Searle et al., 2016; Ranadive et al., 2017). Molecular methods for malaria detection and species identification such as nested polymerase chain reaction (nPCR), quantitative PCR (qPCR), reverse transcriptase quantitative polymerase chain reaction (RTqPCR), and nucleic acid sequence-based amplification (NASBA) can contribute to better understanding of the malaria infections that infect mosquitoes by quantification of the relative contribution of patent and sub patent infections to the human infectious

reservoir (Bousema & Drakeley, 2017). Since RDTs are instrumental to malaria surveillance programs, their diagnostic performance should be systematically monitored over time using sensitive and highly specific methods detecting *Plasmodium* spp. molecular markers using molecular tools mentioned above (Hosch et al., 2022).

Molecular screening of blood samples from suspected malaria cases by PCR or qPCR can inform on the proportion of clinical infections missed by routine diagnosis at health posts which should include looking at both RDT positives and 10% of RDT negatives to detect how many low parasitaemia infections are missed when doing RDT based RACD (Tambo et al., 2018). PCR screening of blood samples collected in the frame of mass blood surveys is required to understand the prevalence and density of asymptomatic infections, and associated risk factors (Holzschuh & Koepfli, 2022). Real-time quantitative PCR is important for detecting and quantifying parasite density including sub-patent low density infections. This technique is more sensitive than the conventionally used diagnostic methods like microscopy and RDTs and can detect low parasitaemia infections (Tedla, 2019).

This study used quantitative PCR to determine parasite density as a proxy for transmission intensity. DNA was extracted from DBS samples using the Chelex method but this could not be successfully used to quantify the DNA in the samples. The Chelex extracted samples would show very high Ct values or none at all even though the samples had strong bands with gel electrophoresis. Although genomic DNA isolation using the Chelex 100 resin is rapid and inexpensive, the DNA obtained by this method has a low concentration in solution and contains suspended debris and impurities that when amplified by more sensitive qPCR assays lead to high efficiencies (Singh, Kumari & Iyengar, 2018). The presence of debris in the DNA solution may

result in degradation of DNA on long term storage and inhibition of the quantitative polymerase chain reaction (Singh, Kumari & Iyengar, 2018). The Chelex method was efficient in confirming and identifying through conventional PCR the *P. falciparum* species from the 589 DBS samples that were RDT positive. The Chelex method was cost effective and acted as a filter in determining which samples would be used for extraction with the QIAamp DNA extraction kit which yields pure DNA for further downstream, more sensitive PCR applications such as qPCR in this instance.

It is important to understand the contributions of symptomatic and asymptomatic infections to onward transmission of malaria parasites to mosquitoes as it is essential for guiding interventions to reduce and eliminate transmission. These contributions depend on parasite density, infection duration, care-seeking behaviour, and importantly gametocyte production (Andolina et al., 2021). Quantification of parasitaemia from the 2017 outbreak samples showed that there was a mean parasite density of 46088 p/µL, which was significantly higher than the mean parasite density of samples from the 2021 outbreak season (35211 p/µL) (Mann-Whitney test, p=0.008). This was despite that there were less cases (4151) in 2017 than the malaria cases (10191) in the 2021 malaria outbreak. The second hypothesis for this study was that parasitaemia can be used as an indicator for transmission intensity, higher parasitaemia is associated with higher malaria transmission intensity and risk. This was confirmed by Spearman's correlation coefficient for both outbreaks however more information from molecular analysis of samples is required to make more qualified inferences about the link between parasitaemia and transmission intensity.

Multiplicity of infection (MOI) which describes the number of different Plasmodia clones infecting a patient, and the heterozygosity of infections are linked with transmission intensity as they both increase with greater transmission intensity. These

parameters have also been used to show that low transmission settings can take on parameters similar to those of a geographically neighbouring high transmission setting due to importation. A study by Guiguemde et al., (2020) showed that population movement from high transmission areas to low transmission areas was a factor which contributed to increased malaria transmission (Guiguemde et al., 2020). In 2017, number of cases was only 4151 cases, lower than in 2021, but parameters such as parasitaemia, MOI and heterozygosity may have been similar to those of the Kavango region due to importation, which could explain the high parasitaemia in 2017. In 2017, Kavango region had the highest number of cases and a study by Tessema et al., (2019) showed that in 2019, 40% of malaria cases in Zambezi region were linked to those in Kavango East region (Tessema et al., 2019). Importation can affect the molecular parameters of parasite populations and more data from analysis may be required to complement parasite density in determining transmission intensity. Nevertheless, it is clear that there is a correlation between parasite density and severity of a malaria outbreak. The outbreak in 2021 in Zambezi region was more severe than that in 2017 and could be due to the COVID-19 pandemic where attention was diverted more towards COVID-19 than malaria affecting case management. The pandemic could have also contributed to the high malaria cases due to reduced control efforts (where spray coverage was below the WHO recommendations) resulting in more cases (NVDCP, 2021). COVID-19 reduced movement of people thus this outbreak could have been mostly due to locally acquired infections which was more reflective of lower transmission intensity.

The study hypothesized that high parasitaemia in malaria positive cases will contribute to higher gametocyte carriage and hence high malaria transmission risk. The results from the 2017 and 2021 outbreak samples show that there is a moderate and strong

correlation between parasite density and gametocyte carriage, as parasitaemia density increased, it greatly increased the likelihood of gametocytaemia which confirms the third hypothesis in this study. Higher densities of asexual stage malaria parasites are commonly associated with the presentation of symptoms. Since gametocytes develop from asexual parasites, individuals with clinical malaria and higher parasite densities may have more gametocytes and are more likely to infect mosquitoes (Barry et al., 2021). Multi-genotype malaria infections are frequent in endemic areas, and people commonly harbour several genetically distinct *Plasmodium falciparum* variants. However, the influence of genetic multiplicity and whether some specific genetic variants are more or less likely to invest into gametocyte production is not clearly understood (Sondo et al., 2021).

A closer look revealed that samples from the 2017 outbreak had higher parasitaemia (46088 p/µL) and lower gametocytaemia (116 g/µL) whereas the 2021 outbreak had lower parasitaemia (35211 p/µL) and higher gametocytaemia (892 g/µL). The difference in gametocytaemia could be due parasite clones in the 2021 outbreak having a strain that outcompeted other strains creating selective pressure for them to form gametocytes (Gwarinda et al., 2021). According to the literature, gametocyte formation occurs as a stress response of the parasite which allows it to escape from an increasingly unfavourable environment (Rakotoarisoa et al., 2022). Several environmental factors including drugs, host cells and haemoglobin concentrations have been proposed to lead to sexual commitment of asexual blood stage parasites (Ngotho et al., 2019).

Some antimalarial drugs have shown to be effective against viruses, which include chloroquine derived from the bark of Cinchona tree, artemisinin from Artemisia annua and neem tree (Azadirachta indica/dogoyaro) (Muanya, 2022). The 2021 outbreak

occurred during the COVID-19 pandemic where many therapeutic interventions were used including herbal medicines which may have stressed parasites so they formed gametocytes. Other studies have shown that medicinal plants have properties that contribute to anti-inflammatory (du Preez-Bruwer, Mumbengegwi & Louw, 2022) and anti-plasmodial activity (Preez, Bussel & Mumbengegw, 2017).

Some studies demonstrated that clonal multiplicity can promote both the longevity of *P. falciparum* infection in patients and their ability to produce gametocytes (Ayanful-Torgby et al., 2016). Genetic diversity in an area may affect parasite densities and molecular studies have shown that low genetic diversity is linked with low transmission intensity (Gwarinda et al., 2021). This suggests that after people are infected once, subsequent infections contain antigenically very similar parasites and may therefore be easier to control (Okell et al., 2012). Accordingly, positive relationship between MOI and gametocyte carriage has been reported and can translate into a positive correlation between MOI and mosquito infection rates (Sondo et al., 2021). Therefore, this positive association between MOI and gametocyte carriage may suggest that infections with multiple clones have simply more chance to contain some clones that will evade the host immune response, persist in the host and result in gametocyte development (Sondo et al., 2021). Genomic analysis can contribute to a better understanding of parasite transmission networks, genetic diversity of parasites and their ability to form gametocytes as well as the MOI (Ayanful-Torgby et al., 2016).

An interesting pattern from the box plots was observed for the medians for both outbreaks in the Zambezi region where the low-density *Plasmodium* infections had higher gametocytes than the moderate infections. This finding is consistent with a study by Salgado et al., (2021) which showed that some individuals with very low parasite densities were noted to have unexpectedly high gametocyte densities.

However, in this study, statistically, there was no difference between gametocytaemia from low and moderate parasitaemia where p=0.910, showing that it was greater than 0.05 confirming that there is no significant difference between gametocytaemia from low and moderate parasitaemia. This is an important finding because low density infections are sometimes not detected by RDTs and are neglected, yet they have the potential for onward transmission. The fact that there was no difference in gametocytaemia between low and moderate parasitaemia shows that low density infections are important as they equally contribute to transmission risk as they harbour gametocytes as well. This underlines the importance of use of more sensitive molecular tools with targeted interventions such as RACD, MSAT as well as the use of rfMDA in targeting high-transmission risk groups (Roberts et al., 2021). These groups could be the potential reason and source for onward transmission during the outbreaks when mosquito populations increase during the rainy season. On the surface, this might suggest that treating high density infections in high-transmission settings would contribute to the overall reduction of gametocyte carriage, however such a strategy neglects the potential contribution of sub-microscopic infections (Salgado et al., 2021). Therefore, it is also important to look at the parasite distribution during the dry seasons (out of season) and observe parasitaemia and gametocytaemia and how they compare to outbreaks. Pfhrp2 and pfhrp3 (pfhrp2/3) may be deleted in some parasites rendering them undetectable by pfhrp2/3 - based RDTs. This loss of efficacy can lead to untreated malaria cases, thus compromising malaria case management and control possibly facilitating onward transmission (Nundu et al., 2022). The WHO recommends continuous nationwide surveillance of potential parasites harbouring pfhrp2/3 deletions and if their prevalence exceeds 5%, it is recommended that alternative RDTs should be used (Nundu et al., 2022). Prevalence

of pfhrp2/3 deletions in Namibia should also be further studied using RDT negatives. Therefore, 10% of the negative RDTs should be tested with molecular tools for quality assurance to detect any potential low-density infections or deletions, as this study showed that that low-density infections are equally important in facilitating onward transmission. For the 2017 outbreak samples, no statistics were computed by SPSS as there were not enough valid cases to perform the Median Test for Gametocytaemia 2017 * Parasitaemia 2017, therefore, a larger sample size is needed to power the study.

There are numerous and complex reasons for Namibia missing its elimination target of 2022 (Jacobson et al., 2019) such as possibly not detecting all cases due to people with fever or flu-like symptoms delaying to visit healthcare facilities in fear of getting infected with COVID-19 or worried that they had COVID-19 and might pass it on to others. Before the pandemic, Namibia had recorded less malaria cases in 2018-2019, however the next outbreak in 2020-2021 could have been due to resources diverted to deal with COVID-19. The presence of COVID-19 has greatly agitated the health care system in many countries leading to a transient halt in focus on some infectious diseases like malaria, HIV/AIDS and tuberculosis (Oyegoke, Maharaj, et al., 2022). Also delays in procuring or delivery of insecticides and spray pumps resulted in low coverage in 2020 and 2021 before the rain season and this could have resulted in the increased number of cases.

Future focus on studies involving larger sample size, continuous and consistent evaluation of program impacts on malaria positivity and reduction across malaria-endemic regions is suggested. Also improving the already-achieved malaria elimination gains requires the sustenance of the currently effective system with policies that give strong consideration to a wider coverage of malaria programs using

more sensitive, specific and easy-to-use diagnostic methods (Oyegoke, Akoniyon, et al., 2022).

Chapter 6. Conclusion and Recommendations

This study showed the importance of molecular tools in surveillance of malaria in low transmission settings. *P. falciparum* was confirmed to be the major parasite causing malaria outbreaks in Zambezi region. Conventional PCR is a tool that can be used to identify Plasmodia species as well as other important parasite characteristics such as drug resistance and gene deletions techniques. PCR also showed that use of RDTs for RACD do not give an accurate picture of true malaria cases during outbreaks with only 38% of cases detected by RDTs being true-positives, the rest being false-positives due to HRP-2 persistence. Molecular surveillance was conducted on all RDT positive DBS for surveillance of possibility of either persistence, new infection or delayed parasite clearance/resistance. Since majority of the cases were PCR negative and RDT positive, the study can conclude that the false positives are due to persistence. Quantitative PCR was used to show the correlation between parasite density and intensity of infection although the use of other molecular tools for genomic analysis can be used to complement qPCR including measurement of MOI, heterozygosity index and transmission networks. These tools will increase the ability to answer questions such as the contribution of importation to local transmission and transmission intensity, as well as the sources and sinks of the parasites, questions that could not be answered fully by parasite density alone.

Most importantly, RTqPCR showed the correlation of parasite density and gametocyte carriage and that low parasitaemia infections are as likely to carry gametocytes in similar numbers to moderate density infections. This is important not only because RDTs are not likely to detect these low parasitaemia infections due to low sensitivity but also because they carry gametocytes adding to risk of transmission of malaria.

This study was conducted only on RDT positive samples using PCR, however, it leaves a gap on false negative RDT results which might result in loss of information about malaria cases. It is therefore recommended that; PCR analysis is also conducted on at least 10% of RDT negative samples to quantify this risk. This study showed that estimating the contribution of low parasitaemia infections to malaria transmission should be a priority in the malaria elimination era. For RACD to be effective, deployment of molecular tools that can detect gametocyte levels in microscopically undetectable infections should be a part of malaria surveillance. Detection of this sub-group may be important in reducing residual transmission during outbreaks and during the offseason. The use of these tools help to improve the understanding of malaria epidemiology to improve decision making and help Namibia achieve malaria elimination. It is recommended that a similar analysis be done during the non-malaria season to determine parasite parameters such as presence, density, MOI, gametocyte carriage to help understand how they contribute to transmission on the onset of the malaria season.

Northern Namibia has experienced outbreaks in the last 5 years stalling malaria elimination efforts, the drivers for the outbreaks have been due to importation, environmental conditions such as rainfall, vector behaviour and coverage of malaria interventions. The heterogeneity of malaria transmission requires tailoring of cost-effective interventions and not a one-size fits all blanket intervention.

It is recommended that:

- Molecular surveillance using tools similar to those in this study, be instituted at sentinel sites (clinics as passive surveillance) and be employed to understand these drivers of sustained transmission. This will enable effective, tailor-made interventions to be developed for implementation.

- Molecular tools to be used as part of Reactive Case Detection and Mass Screen and Treat approaches during outbreaks to characterize parasites and determine their epidemiology so this information can be used to mitigate against transmission.
- Molecular tools to be used when targeting interventions to high-risk groups such as children, cattle herders and agricultural workers who may have poor treatment seeking behaviour and be transmitting malaria asymptomatically for more accurate profiling of risk of transmission.
- Molecular tools and genomic analysis to be used as part of malaria surveillance beyond transmission intensity and risk of transmission, to gain insights into characteristics of parasites such as drug resistance; parasite relatedness, transmission networks; sensitivity to diagnosis by RDTs due to pfhpr2/3 gene deletion and parasite origins (local *versus* imported and parasite sources and sinks).

The WHO recommended, routinely used tools – microscopy and RDTs have contributed greatly and are still contributing to the reduction in malaria morbidity and mortality, however as the malaria parasite and mosquito vector find different ways to evade the interventions used, new, improved and more sensitive methods, tools and interventions will be needed to reach and achieve malaria elimination.

Chapter 7. References

Abebaw, A., Aschale, Y., Kebede, T. & Hailu, A. 2022. The prevalence of symptomatic and asymptomatic malaria and its associated factors in Debre Elias district communities , Northwest Ethiopia. *Malaria Journal*. 1–10. DOI: 10.1186/s12936-022-04194-7.

Acharya, P., Garg, M., Kumar, P., Munjal, A. & Raja, K.D. 2017. Host – Parasite Interactions in Human Malaria : Clinical Implications of Basic Research. 8(May):1–16. DOI: 10.3389/fmicb.2017.00889.

Adedeji, E.O., Ogunlana, O.O., Fatumo, S., Beder, T., Ajamma, Y., Koenig, R. & Adebiyi, E. 2020. Anopheles metabolic proteins in malaria transmission, prevention and control: A review. *Parasites and Vectors*. 13(1):1–30. DOI: 10.1186/s13071-020-04342-5.

Adhikari, S.P., Meng, S., Wu, Y., Mao, Y., Ye, R., Wang, Q., Sun, C., Sylvia, S., et al. 2020. Epidemiology, causes, clinical manifestation and diagnosis, prevention and control of COVID during the early outbreak period. *Infectious Diseases of Poverty*. 9(29):1–12.

Agbana, H.B., Rogier, E., Lo, A., Abukari, Z., Jones, S., Gyan, B., Aidoo, M. & Amoah, L.E. 2022. Detecting asymptomatic carriage of Plasmodium falciparum in southern Ghana : utility of molecular and serological diagnostic tools. *Malaria Journal*. 1–11. DOI: 10.1186/s12936-022-04078-w.

Ajayi, I.O., Ajumobi, O.O. & Falade, C. 2020. Malaria and COVID-19: commonalities, intersections and implications for sustaining malaria control Ikeoluwapo. *Pan African Medical Journal*. 37(Supp 1):1–10.

Akala, H.M., Watson, O.J., Mitei, K.K., Juma, D.W., Verity, R., Ingasia, L.A., Opot,

B.H., Okoth, R.O., et al. 2021. Plasmodium interspecies interactions during a period of increasing prevalence of Plasmodium ovale in symptomatic individuals seeking treatment: an observational study. *The Lancet Microbe*. 2(4):e141–e150. DOI: 10.1016/S2666-5247(21)00009-4.

Andolina, C., Rek, J.C., Briggs, J., Okoth, J., Musiime, A., Ramjith, J., Teyssier, N., Conrad, M., et al. 2021. Sources of persistent malaria transmission in a setting with effective malaria control in eastern Uganda : a longitudinal , observational cohort study. *The Lancet Infectious Diseases*. 21(11):1568–1578. DOI: 10.1016/S1473-3099(21)00072-4.

Apinjoh, T.O., Ntasin, V.N., Tataw, P.C.C., Ntui, V.N., Njimoh, D.L., Ngwa, F.C. & Achidi, E.A. 2021. Comparison of conventional and non - invasive diagnostic tools for detecting Plasmodium falciparum infection in southwestern Cameroon : a cross - sectional study. 1–11. DOI: 10.1186/s40249-021-00859-8.

Ashley, E.A., Recht, J. & White, N.J. 2014. Primaquine: The risks and the benefits. *Malaria Journal*. 13(1):1–7. DOI: 10.1186/1475-2875-13-418.

Ashton, R.A., Prosnitz, D., Andrada, A., Herrera, S. & Yé, Y. 2020. Evaluating malaria programmes in moderate - and low - transmission settings : practical ways to generate robust evidence. *Malaria Journal*. 1–14. DOI: 10.1186/s12936-020-03158-z.

Autino, B., Noris, A., Russo, R. & Castelli, F. 2012. Epidemiology of malaria in endemic areas. *Mediterranean Journal of Hematology and Infectious Diseases*. 4(1). DOI: 10.4084/MJHID.2012.060.

Ayanful-Torgby, R., Oppong, A., Abankwa, J., Acquah, F., Williamson, K.C. & Amoah, L.E. 2016. Plasmodium falciparum genotype and gametocyte prevalence in

children with uncomplicated malaria in coastal Ghana. *Malaria Journal*. 15(1):1–10. DOI: 10.1186/s12936-016-1640-8.

Bachman, C.M., Cate, D.M., Grant, B., Burkot, S., Mulondo, J., Hsieh, H. V., Chamai, M., Odongo, B., et al. 2022. A Novel Malaria Lateral Flow Assay for Detecting Plasmodium falciparum Lactate Dehydrogenase in Busia, Uganda. *American Journal of Tropical Medicine and Hygiene*. 106(3):850–852. DOI: 10.4269/ajtmh.21-0956.

Baker, D.A. 2010. Malaria gametocytogenesis. *Molecular and Biochemical Parasitology*. 172(2):57–65. DOI: 10.1016/j.molbiopara.2010.03.019.

Bamou, R., Nematchoua-weyou, Z., Lontsi-demano, M., Defo-talom, B.A., Gilberine, L., Ad, M., Paul, M. & Mayi, A. 2021. Heliyon Performance assessment of a widely used rapid diagnostic test CareStart ™ compared to microscopy for the detection of Plasmodium in asymptomatic patients in the Western region of Cameroon. 7(February). DOI: 10.1016/j.heliyon.2021.e06271.

Barbé, B., Gillet, P., Beelaert, G., Fransen, K. & Jacobs, J. 2012. Assessment of desiccants and their instructions for use in rapid diagnostic tests. 1–10. DOI: 10.1186/1475-2875-11-326.

Barry, A., Bradley, J., Stone, W., Guelbeogo, M.W., Lanke, K., Ouedraogo, A., Soulama, I., Nébié, I., et al. 2021. Higher gametocyte production and mosquito infectivity in chronic compared to incident Plasmodium falciparum infections. *Nature Communications*. DOI: 10.1038/s41467-021-22573-7.

Belete, T.M. 2020. Recent progress in the development of new antimalarial drugs with novel targets. *Drug Design, Development and Therapy*. 14:3875–3889. DOI: 10.2147/DDDT.S265602.

Berzosa, P., De Lucio, A., Romay-Barja, M., Herrador, Z., González, V., García, L., Fernández-Martínez, A., Santana-Morales, M., et al. 2018. Comparison of three diagnostic methods (microscopy, RDT, and PCR) for the detection of malaria parasites in representative samples from Equatorial Guinea 11 Medical and Health Sciences 1108 Medical Microbiology. *Malaria Journal*. 17(1):1–12. DOI: 10.1186/s12936-018-2481-4.

Bio Rad. 2006. Real time PCR Applications Guide. 36.

Boonbanjong, P., Treerattrakoon, K., Waiwinya, W., Pitikultham, P. & Japrung, D. 2022. Isothermal Amplification Technology for Disease Diagnosis. *Biosensors*. 12(9). DOI: 10.3390/bios12090677.

Bousema, T. & Drakeley, C. 2017. Determinants of Malaria Transmission at the Population Level. 1–14.

Bousema, J.T., Gouagna, L.C., Drakeley, C.J., Meutstege, A.M., Okech, B.A., Akim, I.N.J., Beier, J.C., Githure, J.I., et al. 2004. Plasmodium falciparum gametocyte carriage in asymptomatic children in western Kenya. *Malaria Journal*. 3:1–6. DOI: 10.1186/1475-2875-3-18.

Bria, Y.P., Yeh, C. & Beding, S. 2021. International Journal of Infectious Diseases Signi fi cant symptoms and nonsymptom-related factors for malaria diagnosis in endemic regions of Indonesia. 103:194–200. DOI: 10.1016/j.ijid.2020.11.177.

Britton, S., Cheng, Q. & McCarthy, J.S. 2016. Novel molecular diagnostic tools for malaria elimination: A review of options from the point of view of high-throughput and applicability in resource limited settings. *Malaria Journal*. 15(1):1–8. DOI: 10.1186/s12936-016-1158-0.

Burkot, T.R., Farlow, R., Min, M., Espino, E., Mnzava, A. & Russell, T.L. 2019. A global analysis of National Malaria Control Programme vector surveillance by elimination and control status in 2018. *Malaria Journal*. 18(1):1–12. DOI: 10.1186/s12936-019-3041-2.

Capela, R., Moreira, R. & Lopes, F. 2019. An overview of drug resistance in protozoal diseases. *International Journal of Molecular Sciences*. 20(22). DOI: 10.3390/ijms20225748.

CDC. 2019. Centers for Disease Control and Prevention DPDx - Laboratory Identification of Parasites of Public Health Concern Enterobiasis. *Cdc*. 1–6.

Chipwaza, B. & Sumaye, R.D. 2020. High malaria parasitemia among outpatient febrile children in low endemic area , East - Central Tanzania in 2013. *BMC Research Notes*. 4–9. DOI: 10.1186/s13104-020-05092-4.

Chourasia, M.K., Raghavendra, K., Bhatt, R.M., Swain, D.K., Meshram, H.M., Meshram, J.K., Suman, S., Dubey, V., et al. 2017. Additional burden of asymptomatic and sub-patent malaria infections during low transmission season in forested tribal villages in Chhattisgarh, India. *Malaria Journal*. 16(1):1–9. DOI: 10.1186/s12936-017-1968-8.

Chung, A.M., Love, E., Neidel, J., Mendai, I., Nairenge, S., Wyk, L. Van, Rossi, S., Larson, E., et al. 2022. Strengthening Management , Community Engagement , and Sustainability of the Subnational Response to Accelerate Malaria Elimination in Namibia. 106(6):1646–1652. DOI: 10.4269/ajtmh.21-1195.

Coleman, A.N. 2022. Rethinking district-level governance of malaria in Ghana : A narrative review. 1–15. DOI: 10.1371/journal.pgph.0000764.

Cotter, C., Sudathip, P., Herdiana, H., Cao, Y., Liu, Y., Luo, A., Ranasinghe, N., Bennett, A., et al. 2017. Piloting a programme tool to evaluate malaria case investigation and reactive case detection activities: Results from 3 settings in the Asia Pacific. *Malaria Journal*. 16(1):1–13. DOI: 10.1186/s12936-017-1991-9.

Cox, F.E.G. 2010. History of the discovery of the malaria parasites and their vectors. (Figure 1):1–9.

Dahal, P., Khanal, B., Rai, K., Kattel, V., Yadav, S. & Bhattarai, N.R. 2021. Challenges in Laboratory Diagnosis of Malaria in a Low-Resource Country at Tertiary Care in Eastern Nepal: A Comparative Study of Conventional vs. Molecular Methodologies. *Journal of Tropical Medicine*. 2021. DOI: 10.1155/2021/3811318.

Das, D., Dahal, P., Dhorda, M., Citarella, B.W., Kennon, K., Stepniewska, K., Felger, I., Chappuis, F., et al. 2021. Review Article: A systematic literature review of microscopy methods reported in malaria clinical trials. *American Journal of Tropical Medicine and Hygiene*. 104(3):836–841. DOI: 10.4269/ajtmh.20-1219.

Das, S., Peck, R.B., Barney, R., Jang, I.K., Kahn, M., Zhu, M. & Domingo, G.J. 2018. Performance of an ultra - sensitive Plasmodium falciparum HRP2 - based rapid diagnostic test with recombinant HRP2 , culture parasites , and archived whole blood samples. *Malaria Journal*. 1–7. DOI: 10.1186/s12936-018-2268-7.

Datoo, M.S., Natama, H.M., Somé, A., Bellamy, D., Traoré, O., Rouamba, T., Tahita, M.C., Ido, N.F.A., et al. 2022. Efficacy and immunogenicity of R21/Matrix-M vaccine against clinical malaria after 2 years' follow-up in children in Burkina Faso: a phase 1/2b randomised controlled trial. *The Lancet. Infectious diseases*. DOI: 10.1016/S1473-3099(22)00442-X.

Dausab, L.L. 2018. AN INVESTIGATION INTO THE PREVALENCE OF POINT

MUTATIONS ASSOCIATED WITH ANTIMALARIAL DRUG RESISTANCE IN PLASMODIUM FALCIPARUM FOUND IN THE ZAMBEZI REGION OF NAMIBIA. Research Thesis. (March).

Deelder, W., Benavente, E.D., Phelan, J., Manko, E., Campino, S., Palla, L. & Clark, T.G. 2021. Using deep learning to identify recent positive selection in malaria parasite sequence data. *Malaria Journal*. 20(1):1–9. DOI: 10.1186/s12936-021-03788-x.

Delahunt, C.B., Jaiswal, M.S., Horning, M.P., Janko, S., Thompson, C.M., Kulhare, S., Hu, L., Ostbye, T., et al. 2019. Fully-automated patient-level malaria assessment on field-prepared thin blood film microscopy images. *2019 IEEE Global Humanitarian Technology Conference, GHTC 2019*. (i). DOI: 10.1109/GHTC46095.2019.9033083.

Dhiman, S. 2019. Are malaria elimination efforts on right track? An analysis of gains achieved and challenges ahead. *Infectious Diseases of Poverty*. 1–19.

Dhorda, M., Ba, E.H., Kevin Baird, J., Barnwell, J., Bell, D., Carter, J.Y., Dondorp, A., Ekawati, L., et al. 2020. Towards harmonization of microscopy methods for malaria clinical research studies. *Malaria Journal*. 19(1). DOI: 10.1186/s12936-020-03352-z.

Duffy, P.E. & Patrick Gorres, J. 2020. Malaria vaccines since 2000: progress, priorities, products. *npj Vaccines*. 5(1):1–9. DOI: 10.1038/s41541-020-0196-3.

Egbewande, O.M. 2022. Public Health in Practice The RTS , S malaria vaccine : Journey from conception to recommendation. *Public Health in Practice*. 4(June):100283. DOI: 10.1016/j.puhip.2022.100283.

Elimination 8. 2015. Elimination 8 Strategic Plan :2015-2020.

Elimination 8. 2018. About the Elimination 8. (April).

Elimination 8. 2019. ANNUAL Report 2019. 1–52.

Elimination 8. 2021. ANNUAL REPORT 2021 SADC MALARIA ELIMINATION EIGHT.

Fitri, L.E., Widaningrum, T., Endharti, A.T., Hatta, M., Nuning, P., Yudhinata, R., Nugraha, B. & D, B.A.C.K.G.R.O.U.N. 2022. Malaria diagnostic update : From conventional to advanced method. (December 2020):1–14. DOI: 10.1002/jcla.24314.

Fornace, K.M., Diaz, A. V, Lines, J. & Drakeley, C.J. 2021. Achieving global malaria eradication in changing landscapes. *Malaria Journal*. 1–14. DOI: 10.1186/s12936-021-03599-0.

Galatas, B., Mayor, A., Gupta, H., Balanza, N., Jang, I.K., Nhamussua, L., Simone, W., Cisteró, P., et al. 2020. Field performance of ultrasensitive and conventional malaria rapid diagnostic tests in southern Mozambique. *Malaria Journal*. 1–15. DOI: 10.1186/s12936-020-03526-9.

Gatton, M.L., Ciketic, S., Barnwell, J.W., Cheng, Q., Chiodini, P.L., Incardona, S., Bell, D., Cunningham, J., et al. 2018. An assessment of false positive rates for malaria rapid diagnostic tests caused by non- Plasmodium infectious agents and immunological factors. 1–9.

Gillet, P., Mori, M., Esbroeck, M. Van, Ende, J. Van Den & Jacobs, J. 2009. Assessment of the prozone effect in malaria rapid diagnostic tests. 7:1–7. DOI: 10.1186/1475-2875-8-271.

Gosling, R., Chimumbwa, J., Uusiku, P., Rossi, S., Ntuku, H., Harvard, K., White,

C., Tatarsky, A., et al. 2020. District-level approach for tailoring and targeting interventions: A new path for malaria control and elimination. *Malaria Journal*. 19(1):1–7. DOI: 10.1186/s12936-020-03185-w.

Graumans, W., Jacobs, E., Bousema, T. & Sinnis, P. 2020. When Is a Plasmodium-Infected Mosquito an Infectious Mosquito? *Trends in Parasitology*. 36(8):705–716. DOI: 10.1016/j.pt.2020.05.011.

Grossenbacher, B., Holzschuh, A., Hofmann, N.E., Omar, K.A., Stuck, L., Fakih, B.S., Ali, A., Yukich, J., et al. 2020. Molecular methods for tracking residual Plasmodium falciparum transmission in a close - to - elimination setting in Zanzibar. *Malaria Journal*. 1–12. DOI: 10.1186/s12936-020-3127-x.

Gruenberg, M., Moniz, C.A., Hofmann, N.E., Koepfli, C., Robinson, L.J., Nate, E., Monteiro, W.M., Melo, G.C. De, et al. 2020. Utility of ultra - sensitive qPCR to detect Plasmodium falciparum and Plasmodium vivax infections under different transmission intensities. *Malaria Journal*. 1–10. DOI: 10.1186/s12936-020-03374-7.

Gueye, C.S., Gerigk, M., Newby, G., Lourenco, C., Uusiku, P. & Liu, J. 2014. Namibia ' s path toward malaria elimination : a case study of malaria strategies and costs along the northern border. 1–16.

Guiguemde, K.T., Dieye, Y., Lô, A.C., Ndiaye, M., Lam, A., Manga, I.A., Sow, G.D., Diop, M., et al. 2020. Molecular detection and quantification of Plasmodium falciparum gametocytes carriage in used RDTs in malaria elimination settings in northern Senegal. *Malaria Journal*. 1–7. DOI: 10.1186/s12936-020-03204-w.

Guirou, E.A., Schindler, T., Hosch, S., Donfack, O.T., Yoboue, C.A., Krähenbühl, S., Deal, A., Cosi, G., et al. 2020. Molecular malaria surveillance using a novel protocol for extraction and analysis of nucleic acids retained on used rapid diagnostic

tests. *Scientific Reports*. 10(1):1–14. DOI: 10.1038/s41598-020-69268-5.

Gwarinda, H.B., Tessema, S.K., Raman, J., Greenhouse, B. & Birkholtz, L.M. 2021. Parasite genetic diversity reflects continued residual malaria transmission in Vhembe District, a hotspot in the Limpopo Province of South Africa. *Malaria Journal*. 20(1):1–13. DOI: 10.1186/s12936-021-03635-z.

Haindongo, E. 2016. Investigation into the risk factors for Malaria transmission in the Omusati region. (July).

Haiyambo, D.H., Uusiku, P., Mumbengegwi, D., Pernica, J.M., Bock, R., Malleret, B., Rénia, L., Greco, B., et al. 2019. Molecular detection of P. Vivax and P. ovale foci of infection in asymptomatic and symptomatic children in Northern Namibia. *PLoS Neglected Tropical Diseases*. 13(5):1–11. DOI: 10.1371/journal.pntd.0007290.

Hakizimana, D., Ntizimira, C., Mbituyumuremyi, A., Hakizimana, E., Mahmoud, H., Birindabagabo, P., Musanabaganwa, C. & Gashumba, D. 2022. The impact of Covid - 19 on malaria services in three high endemic districts in Rwanda : a mixed - method study. *Malaria Journal*. 1–17. DOI: 10.1186/s12936-022-04071-3.

Hammami, I., Nuel, G. & Garcia, A. 2013. Statistical Properties of Parasite Density Estimators in Malaria. *PLoS ONE*. 8(3). DOI: 10.1371/journal.pone.0051987.

Henry, N.B., Sermé, S.S., Siciliano, G., Sombié, S., Diarra, A., Sagnon, N., Traoré, A.S., Sirima, S.B., et al. 2019. Biology of Plasmodium falciparum gametocyte sex ratio and implications in malaria parasite transmission. *Malaria Journal*. 18(1):1–8. DOI: 10.1186/s12936-019-2707-0.

Hofmann, N., Mwingira, F., Shekalaghe, S., Robinson, L.J., Mueller, I. & Felger, I. 2015. Ultra-Sensitive Detection of Plasmodium falciparum by Amplification of

Multi-Copy Subtelomeric Targets. *PLoS Medicine*. 12(3):1–21. DOI: 10.1371/journal.pmed.1001788.

Holzschuh, A. & Koepfli, C. 2022. Tenfold difference in DNA recovery rate : systematic comparison of whole blood vs . dried blood spot sample collection for malaria molecular surveillance. *Malaria Journal*. 1–9. DOI: 10.1186/s12936-022-04122-9.

Hopkins, H., Bebell, L., Kambale, W., Dokomajilar, C., Rosenthal, P.J. & Dorsey, G. 2008. Rapid diagnostic tests for malaria at sites of varying transmission intensity in Uganda. *Journal of Infectious Diseases*. 197(4):510–518. DOI: 10.1086/526502.

Horning, M.P., Delahunt, C.B., Bachman, C.M., Luchavez, J., Luna, C., Hu, L., Jaiswal, M.S., Thompson, C.M., et al. 2021. Performance of a fully-automated system on a WHO malaria microscopy evaluation slide set. *Malaria Journal*. 20(1):1–11. DOI: 10.1186/s12936-021-03631-3.

Hosch, S., Yoboue, C.A., Donfack, O.T., Guirou, E.A., Dangy, J.P., Mpina, M., Nyakurungu, E., Blöchliger, K., et al. 2022. Analysis of nucleic acids extracted from rapid diagnostic tests reveals a significant proportion of false positive test results associated with recent malaria treatment. *Malaria Journal*. 1–12. DOI: 10.1186/s12936-022-04043-7.

Hsiang, M.S., Ntshalintshali, N., Dufour, M.K., Dlamini, N., Nhlabathi, N., Vilakati, S., Malambe, C., Zulu, Z., et al. 2020. Active Case Finding for Malaria : A 3-Year National Evaluation of Optimal Approaches to Detect Infections and Hotspots Through Reactive Case Detection in the Low-transmission Setting of Eswatini. 70:10–13. DOI: 10.1093/cid/ciz403.

Hsiang, M.S., Ntuku, H., Roberts, K.W., Dufour, M.K., Whittemore, B., Tambo, M.,

Mccreesh, P., Medzihradsky, O.F., et al. 2020. Effectiveness of reactive focal mass drug administration and reactive focal vector control to reduce malaria transmission in the low malaria-endemic setting of Namibia : factorial design trial. *The Lancet*. 395(10233):1361–1373. DOI: 10.1016/S0140-6736(20)30470-0.

Hsu, B., Sherina, V. & McCall, M.N. 2020. Autoregressive modeling and diagnostics for qPCR amplification. *Bioinformatics*. 36(22–23):5386–5391. DOI: 10.1093/bioinformatics/btaa1000.

Id, A.B.B., Nankabirwa, J.I., Yeka, A., Nsobya, S., Gresty, K., Anderson, K., Mbaka, P., Prosser, C., et al. 2020. Limitations of rapid diagnostic tests in malaria surveys in areas with varied transmission intensity in Uganda 2017-2019 : Implications for selection and use of HRP2 RDTs. DOI: 10.1371/journal.pone.0244457.

Idárraga, L.Z., Rios, A., Cifuentes, V.S., Garzón, E., Castaño, A.T., Takehara, I., Toya, Y., Izuka, M., et al. 2021. Performance of the hematology analyzer XN - 31 prototype in the detection of Plasmodium infections in an endemic region of Colombia. *Scientific Reports*. (0123456789):1–13. DOI: 10.1038/s41598-021-84594-y.

Imwong, M., Hanchana, S., Malleret, B., Rénia, L., Day, N.P.J., Dondorp, A., Nosten, F., Snounou, G., et al. 2014. High-throughput ultrasensitive molecular techniques for quantifying low-density malaria parasitemias. *Journal of Clinical Microbiology*. 52(9):3303–3309. DOI: 10.1128/JCM.01057-14.

Iwuafor, A.A., Ita, O.I., Ogban, G.I., Udoh, U.A. & Amajor, C.A. 2019. Evaluation of Diagnostic Accuracy of Rapid Diagnostic Test for Malaria Diagnosis among Febrile Children in Calabar , Nigeria. DOI: 10.4103/nmj.NMJ.

Jacobson, J.O., Smith, J.L., Cueto, C., Chisenga, M., Roberts, K., Hsiang, M., Gosling, R., Mumbengegwi, D., et al. 2019. Assessing malaria risk at night-time venues in a low-transmission setting: A time-location sampling study in Zambezi, Namibia. *Malaria Journal*. 18(1):1–11. DOI: 10.1186/s12936-019-2807-x.

Jagannathan, P. 2022. Malaria in 2022 : Increasing challenges , cautious optimism. 12–14. DOI: 10.1038/s41467-022-30133-w.

Jiram, A.I., Ooi, C.H., Rubio, J.M., Hisam, S. & Karnan, G. 2019. Evidence of asymptomatic submicroscopic malaria in low transmission areas in Belaga district , Kapit division , Sarawak , Malaysia. *Malaria Journal*. 1–12. DOI: 10.1186/s12936-019-2786-y.

Jong, R.M. De, Tebeje, S.K., Tadesse, F.G., Jore, M.M., Stone, W. & Bousema, T. 2020. Immunity against sexual stage Plasmodium falciparum and Plasmodium vivax parasites. (August 2019):190–215. DOI: 10.1111/imr.12828.

Khanal, P. 2021. Antimalarial and anticancer properties of artesunate and other artemisinins: current development. *Monatshefte fur Chemie*. 152(4):387–400. DOI: 10.1007/s00706-021-02759-x.

Killeen, G.F. 2014. Characterizing, controlling and eliminating residual malaria transmission. *Malaria Journal*. 13(1):1–22. DOI: 10.1186/1475-2875-13-330.

Kipruto, E.K., Ochieng, A.O., Anyona, D.N., Mbalanya, M., Mutua, E.N., Onguru, D., Nyamongo, I.K. & Estambale, B.B.A. 2017. Effect of climatic variability on malaria trends in Baringo County , Kenya. *Malaria Journal*. 1–11. DOI: 10.1186/s12936-017-1848-2.

Koepfli, C. & Yan, G. 2019. Plasmodium Gametocytes in Field Studies: Do we

Measure Commitment to Transmission or Detectability? 34(5):378–387. DOI: 10.1016/j.pt.2018.02.009.Plasmodium.

Koepfli, C., Nguitragool, W., Hofmann, N.E., Robinson, L.J., Ome-Kaius, M., Sattabongkot, J., Felger, I. & Mueller, I. 2016. Sensitive and accurate quantification of human malaria parasites using droplet digital PCR (ddPCR). *Scientific Reports*. 6(1):1–7. DOI: 10.1038/srep39183.

Kosasih, A., Koepfli, C., Dahlan, M.S., Hawley, W.A., Baird, J.K., Mueller, I., Lobo, N.F. & Sutanto, I. 2021. Gametocyte carriage of Plasmodium during mass screening and treatment in West Timor , Indonesia : a longitudinal prospective study. *Malaria Journal*. 1–11. DOI: 10.1186/s12936-021-03709-y.

Kotepui, M., Piwkham, D., PhunPhuech, B., Phiwklam, N., Chupeerach, C. & Duangmano, S. 2015. Effects of malaria parasite density on blood cell parameters. *PLoS ONE*. 10(3):1–11. DOI: 10.1371/journal.pone.0121057.

Kozycki, C.T., Umulisa, N., Rulisa, S., Mwikarago, E.I., Musabyimana, J.P., Habimana, J.P., Karema, C. & Krogstad, D.J. 2017. False - negative malaria rapid diagnostic tests in Rwanda : impact of Plasmodium falciparum isolates lacking hrp2 and declining malaria transmission. *Malaria Journal*. 1–11. DOI: 10.1186/s12936-017-1768-1.

Kuamsab, N., Putaporntip, C., Pattanawong, U. & Jongwutiwes, S. 2012. Simultaneous detection of Plasmodium vivax and Plasmodium falciparum gametocytes in clinical isolates by multiplex-nested RT-PCR. *Malaria Journal*. 11:1–8. DOI: 10.1186/1475-2875-11-190.

Kwenti, T.E., Kwenti, T.D.B., Njunda, L.A., Latz, A., Tufon, K.A. & Nkuo-Akenji, T. 2017. Identification of the Plasmodium species in clinical samples from children

residing in five epidemiological strata of malaria in Cameroon. *Tropical Medicine and Health*. 45(1):1–8. DOI: 10.1186/s41182-017-0058-5.

Lau, Y.L., Lai, M.Y., Fong, M.Y., Jelip, J. & Mahmud, R. 2016. Loop-mediated isothermal amplification assay for identification of five human plasmodium species in Malaysia. *American Journal of Tropical Medicine and Hygiene*. 94(2):336–339. DOI: 10.4269/ajtmh.15-0569.

Lo, E., Zhou, G., Oo, W., Afrane, Y., Githeko, A. & Yan, G. 2015. Low Parasitemia in Submicroscopic Infections Significantly Impacts Malaria Diagnostic Sensitivity in the Highlands of Western Kenya. 1–15. DOI: 10.1371/journal.pone.0121763.

Lourenço, C., Tatem, A.J., Atkinson, P.M., Cohen, J.M., Pindolia, D., Bhavnani, D. & Menach, A. Le. 2019. Strengthening surveillance systems for malaria elimination : a global landscaping of system performance , 2015 – 2017. *Malaria Journal*. 1–11. DOI: 10.1186/s12936-019-2960-2.

M. L. McMorrow, M. Aidoo, S.P.K. 2016. Malaria rapid diagnostic tests in elimination settings—can they find the last parasite? *Physiology & behavior*. 176(3):139–148. DOI: 10.1111/j.1469-0691.2011.03639.x.Malaria.

Manjurano, A., Omolo, J.J., Lyimo, E., Miyaye, D., Kishamawe, C., Matemba, L.E., Massaga, J.J., Changalucha, J., et al. 2021. Performance evaluation of the highly sensitive histidine - rich protein 2 rapid test for Plasmodium falciparum malaria in North - West Tanzania. *Malaria Journal*. 1–9. DOI: 10.1186/s12936-020-03568-z.

Marrelli, M.T. & Brotto, M. 2016. The effect of malaria and anti-malarial drugs on skeletal and cardiac muscles. *Malaria Journal*. 15(1):1–6. DOI: 10.1186/s12936-016-1577-y.

Mathison, B.A. & Pritt, B.S. 2017. crossm Utilization. 55(7):2009–2017.

Mayxay, M., Pukrittayakamee, S., Chotivanich, K., Looareesuwan, S. & White, N.J. 2001. Persistence of Plasmodium falciparum HRP-2 in successfully treated acute falciparum malaria. *Transactions of the Royal Society of Tropical Medicine and Hygiene*. 95(2):179–182. DOI: 10.1016/S0035-9203(01)90156-7.

Mbanefo, A. & Kumar, N. 2020. Evaluation of malaria diagnostic methods as a key for successful control and elimination programs. *Tropical Medicine and Infectious Disease*. 5(2). DOI: 10.3390/tropicalmed5020102.

McCreesh, P., Mumbengegwi, D., Roberts, K., Tambo, M., Smith, J., Whittemore, B., Kelly, G., Moe, C., et al. 2018. Subpatent malaria in a low transmission African setting: A cross-sectional study using rapid diagnostic testing (RDT) and loop-mediated isothermal amplification (LAMP) from Zambezi region, Namibia. *Malaria Journal*. 17(1):1–11. DOI: 10.1186/s12936-018-2626-5.

Meibalan, E. & Marti, M. 2017. Biology of Malaria Transmission. 1–15.

Mobegi, V.A., Herren, J.K., Touray, A.O., Wamunyokoli, F. & Butungi, H. 2021. Prevalence of asymptomatic P. falciparum gametocyte carriage among school children in Mbita, Western Kenya and assessment of the association between gametocyte density, multiplicity of infection and mosquito infection prevalence. *Wellcome Open Research*. 5(May):1–39. DOI: 10.12688/wellcomeopenres.16299.2.

MoHSS. 2022. Ministry of Health and Social Services. Annual Malaria Programme Review and Planning Meeting. (July).

Moonasar, D., Morris, N., Kleinschmidt, I., Maharaj, R., Raman, J., Mayet, N.T., Chb, M.B., Benson, F.G., et al. 2013. F ROM CONTROL TO ELIMINATION What

will move malaria control to elimination in South Africa ? 103(10):801–806. DOI: 10.7196/SAMJ.7445.

Moonasar, D.M., Davies, C., Balawanth, R., Misiani, E., Shandukani, M.B., Raman, J. & Pillay, Y.G. 2021. Progress, challenges and priorities for malaria elimination in South Africa. *Transactions of the Royal Society of South Africa*. 0(0):1–12. DOI: 10.1080/0035919X.2021.1890274.

Morris, U. & Aydin-Schmidt, B. 2021. Performance and Application of Commercially Available Loop-Mediated Isothermal Amplification (LAMP) Kits in Malaria Endemic and Non-Endemic Settings. *Diagnostics*. 11(2):336. DOI: 10.3390/diagnostics11020336.

Mouatcho, J.C. & Goldring, J.P.D. 2013. Malaria rapid diagnostic tests : challenges and prospects. 1491–1505. DOI: 10.1099/jmm.0.052506-0.

Mousa, A., Al-Taiar, A., Anstey, N.M., Badaut, C., Barber, B.E., Bassat, Q., Challenger, J.D., Cunnington, A.J., et al. 2020. The impact of delayed treatment of uncomplicated P. falciparum malaria on progression to severe malaria: A systematic review and a pooled multicentre individual-patient meta-analysis. *PLoS Medicine*. 17(10):1–28. DOI: 10.1371/journal.pmed.1003359.

Muanya. 2022. Antimalarial drugs from plant sources and cure for COVID-19. *https://guardian.ng/features/antimalarial-drugs-from-plant-sources-and-cure-for-covid-19-2/*. 1–19.

Mudare, N., Zingoni, Z.M., Makuwaza, A., Mamini, E., Munyati, S.S., Gwanzura, L., Midzi, N., Mutambu, S.L., et al. 2021. Detecting Plasmodium falciparum in community surveys : a comparison of Paracheck Pf ® Test and ICT Malaria Pf ® Cassette Test to polymerase chain reaction in Mutasa District , Zimbabwe. *Malaria*

Journal. 1–7. DOI: 10.1186/s12936-020-03536-7.

Mukaka, M., Peerawaranun, P., Parker, D.M., Kajeechiwa, L., Nosten, F.H., Nguyen, T.N., Hien, T.T., Tripura, R., et al. 2021. Clustering of malaria in households in the Greater Mekong Subregion : operational implications for reactive case detection. *Malaria Journal*. 1–12. DOI: 10.1186/s12936-021-03879-9.

Mumbengegwi, D.R., Sturrock, H., Hsiang, M., Roberts, K., Kleinschmidt, I., Nghipumbwa, M., Uusiku, P., Smith, J., et al. 2018. Is there a correlation between malaria incidence and IRS coverage in western Zambezi region, Namibia? *Public Health Action*. 8(1):S44–S49. DOI: 10.5588/pha.17.0077.

Mundagowa, P.T. & Chimberengwa, P.T. 2020. Malaria outbreak investigation in a rural area south of Zimbabwe : a case – control study. *Malaria Journal*. 1–10. DOI: 10.1186/s12936-020-03270-0.

Munro, B.A. & McMorran, B.J. 2022. Antimalarial Drug Strategies to Target Plasmodium Gametocytes. *Parasitologia*. 2(2):101–124. DOI: 10.3390/parasitologia2020011.

Murray, C.K., Gasser, R.A., Magill, A.J. & Miller, R.S. 2008. Update on Rapid Diagnostic Testing for Malaria. 21(1):97–110. DOI: 10.1128/CMR.00035-07.

Muthui, M.K., Mogeni, P., Mwai, K., Nyundo, C., Macharia, A., Williams, T.N., Nyangweso, G., Wambua, J., et al. 2019. Gametocyte carriage in an era of changing malaria epidemiology: A 19-year analysis of a malaria longitudinal cohort [version 2; peer review: 2 approved]. *Wellcome Open Research*. 4:1–32. DOI: 10.12688/wellcomeopenres.15186.2.

Mwaiswelo, R.O., Kabuga, H., Kweka, E.J. & Baraka, V. 2022. Is it time for Africa

to adopt primaquine in the era of malaria control and elimination ? *Tropical Medicine and Health*. DOI: 10.1186/s41182-022-00408-5.

Nadeem, A.Y., Shehzad, A., Islam, S.U., Al-suhaimi, E.A. & Lee, Y.S. 2022. Mosquirix ™ RTS , S / AS01 Vaccine Development , Immunogenicity , and Efficacy. 1–15.

Naing, C., Htet, N.H., Aye, S.N., Aung, H.H., Tanner, M. & Whittaker, M.A. 2022. Detection of asymptomatic malaria in Asian countries : a meta - analysis of diagnostic accuracy. *Malaria Journal*. 1–10. DOI: 10.1186/s12936-022-04082-0.

Nalinya, S., Musoke, D. & Deane, K. 2022. Malaria prevention interventions beyond long - lasting insecticidal nets and indoor residual spraying in low - and middle - income countries : a scoping review. *Malaria Journal*. 1–13. DOI: 10.1186/s12936-022-04052-6.

Ndong, I.C., Okyere, D., Enos, J.Y., Mensah, B.A., Nyarko, A., Abuaku, B., Amambua-Ngwa, A., Merle, C.S.C., et al. 2019. Prevalence of asymptomatic malaria parasitaemia following mass testing and treatment in Pakro sub-district of Ghana. *BMC Public Health*. 19(1):1–10. DOI: 10.1186/s12889-019-7986-4.

Nega, D., Id, A.A., Abera, A., Gidey, B. & Tsadik, A.G. 2020. Comprehensive competency assessment of malaria microscopists and laboratory diagnostic service capacity in districts stratified for malaria elimination in Ethiopia. 17(97):1–15. DOI: 10.1371/journal.pone.0235151.

Nghipumbwa, M.H., Ade, S., Kizito, W., Takarinda, K.C., Uusiku, P. & Mumbengegwi, D.R. 2018. *Moving towards malaria elimination: trends and attributes of cases in Kavango region, Namibia, 2010–2014*. V. 8. DOI: 10.5588/pha.17.0076.

Ngotho, P., Soares, A.B., Hentzschel, F., Achcar, F., Bertuccini, L. & Marti, M. 2019. Revisiting gametocyte biology in malaria parasites. *FEMS Microbiology Reviews*. 43(4):401–414. DOI: 10.1093/femsre/fuz010.

Niang, M., Thiam, L.G., Sane, R., Diagne, N., Talla, C., Doucoure, S., Faye, J., Diop, F., et al. 2017. Substantial asymptomatic submicroscopic Plasmodium carriage during dry season in low transmission areas in Senegal: Implications for malaria control and elimination. *PLoS ONE*. 12(8):1–13. DOI: 10.1371/journal.pone.0182189.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N. & Hase, T. 2000. Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research*. 28(12):e63.

Nsanzabana, C. 2019. Strengthening Surveillance Systems for Malaria Elimination by Integrating Molecular and Genomic Data.

Nundu, S.S., Arima, H., Simpson, S. V, Yeddy, B., Chitama, A., Munyeku, Y.B., Muyembe, J.J., Mita, T., et al. 2022. Low prevalence of Plasmodium falciparum parasites lacking pfhrp2 / 3 genes among asymptomatic and symptomatic school - age children in Kinshasa , Democratic Republic of Congo. *Malaria Journal*. 1–10. DOI: 10.1186/s12936-022-04153-2.

NVDCP. 2021. Ministry of Health and Social Services, National Vector-borne Disease Control Programme. (231):65.

NVDCP. 2022. Ministry of Health and Social Services, National Vector-borne Disease Control Programme (NVDCP Programme Updates Presentation). (July).

Oboh, M.A., Oriero, E.C., Ndiaye, T., Badiane, A.S., Ndiaye, D. & Amambua-

Ngwa, A. 2021. Comparative analysis of four malaria diagnostic tools and implications for malaria treatment in southwestern Nigeria. *International Journal of Infectious Diseases*. 108:377–381. DOI: 10.1016/j.ijid.2021.05.049.

Okell, L.C., Bousema, T., Griffin, J.T., Ouédraogo, A.L., Ghani, A.C. & Drakeley, C.J. 2012. Factors determining the occurrence of submicroscopic malaria infections and their relevance for control. *Nature Communications*. 3:1–9. DOI: 10.1038/ncomms2241.

Ouédraogo, A.L., Gonçalves, B.P., Gnémé, A., Wenger, E.A., Guelbeogo, M.W., Ouédraogo, A. & Gerardin, J. 2016. Dynamics of the Human Infectious Reservoir for Malaria Determined by Mosquito Feeding Assays and Ultrasensitive Malaria Diagnosis in Burkina Faso. 213. DOI: 10.1093/infdis/jiv370.

Ouma, W., Id, O., Omondi, C.J., Ochwedo, K.O., Onyango, O., Atieli, H., Id, M.L., Id, C.W., et al. 2022. Risk associations of submicroscopic malaria infection in lakeshore , plateau and highland areas of Kisumu County in western Kenya. 1–16. DOI: 10.1371/journal.pone.0268463.

Oyegoke, O.O., Maharaj, L., Akoniyon, O.P., Kwoji, I., Roux, A.T., Adewumi, T.S., Maharaj, R., Oyebola, B.T., et al. 2022. Malaria diagnostic methods with the elimination goal in view. *Parasitology Research*. 1867–1885. DOI: 10.1007/s00436-022-07512-9.

Oyegoke, O.O., Akoniyon, O.P., Ogunsakin, R.E., Ogunlana, M.O., Adeleke, M.A., Maharaj, R. & Okpeku, M. 2022. A Systematic Review and Meta-Analysis of Malaria Test Positivity Outcomes and Programme Interventions in Low Transmission Settings in Southern Africa , 2000 – 2021. 1–17.

Padilla-Rodríguez, J.C., Olivera, M.J. & Guevara-García, B.D. 2020. Parasite

density in severe malaria in Colombia. *PLoS ONE*. 15(6 June):1–11. DOI: 10.1371/journal.pone.0235119.

Padley, D., Moody, A.H., Chiodini, P.L. & Saldanha, J. 2003. Use of a rapid, single-round, multiplex PCR to detect malarial parasites and identify the species present. *Annals of Tropical Medicine and Parasitology*. 97(2):131–137. DOI: 10.1179/000349803125002977.

Patel, H., Dunican, C. & Cunnington, A.J. 2020. Predictors of outcome in childhood Plasmodium falciparum malaria. *Virulence*. 11(1):199–221. DOI: 10.1080/21505594.2020.1726570.

PathLabs. 2022. Rapid Diagnostic Tests for Malaria. https://www.lalpathlabs.com/blog/rapid-diagnostic-test-for-malaria/. Retrieved on 15 August 2022. 1–5.

Perera, R., Caldera, A. & Wickremasinghe, A.R. 2020. Reactive Case Detection (RACD) and foci investigation strategies in malaria control and elimination : a review. *Malaria Journal*. 1–11. DOI: 10.1186/s12936-020-03478-0.

Picot, S., Cucherat, M. & Bienvenu, A.L. 2020. Systematic review and meta-analysis of diagnostic accuracy of loop-mediated isothermal amplification (LAMP) methods compared with microscopy, polymerase chain reaction and rapid diagnostic tests for malaria diagnosis. *International Journal of Infectious Diseases*. 98:408–419. DOI: 10.1016/j.ijid.2020.07.009.

Praet, N., Asante, K.P., Bozonnat, M.C. & Akité, E.J. 2022. Assessing the safety , impact and effectiveness of RTS , S / AS01 E malaria vaccine following its introduction in three sub - Saharan African countries : methodological approaches and study set - up. *Malaria Journal*. 21(1):1–12. DOI: 10.1186/s12936-022-04144-3.

du Preez-Bruwer, I., Mumbengegwi, D.R. & Louw, S. 2022. In vitro antimalarial properties and chemical composition of Diospyros chamaethamnus extracts. *South African Journal of Botany*. 149:290–296. DOI: 10.1016/j.sajb.2022.06.006.

Preez, I. du, Bussel, R.-J. & Mumbengegw, D. 2017. Evaluation of the Antiplasmodial Properties of Namibian Medicinal Plant Species, Moringa ovalifolia. *Research Journal of Medicinal Plants*. 11(4):167–173. DOI: 10.3923/rjmp.2017.167.173.

Rakotoarisoa, M.A., Fenomanana, J., Dodoson, B.T., Andrianaranjaka, V.H.I. & Ratsimbasoa, A. 2022. Comparative effect of artemether-lumefantrine and artesunate-amodiaquine on gametocyte clearance in children with uncomplicated Plasmodium falciparum malaria in Madagascar. *Malaria Journal*. 21(1):1–7. DOI: 10.1186/s12936-022-04369-2.

Raman, J., Fakudze, P., Sikaala, C.H., Chimumbwa, J. & Raman, J. 2021. Eliminating malaria from the margins of transmission in Southern Africa through the Elimination 8 Initiative. DOI: 10.1080/0035919X.2021.1915410.

Ranadive, N., Kunene, S., Darteh, S., Ntshalintshali, N., Nhlabathi, N., Dlamini, N., Chitundu, S., Saini, M., et al. 2017. Limitations of Rapid Diagnostic Testing in Patients with Suspected Malaria : A Diagnostic Accuracy Evaluation from Swaziland , a Low-Endemicity Country Aiming for Malaria Elimination. 64:12–15. DOI: 10.1093/cid/cix131.

Roberts, C.H., Armstrong, M., Zatyka, E., Boadi, S., Warren, S., Chiodini, P.L., Sutherland, C.J. & Doherty, T. 2013. Gametocyte carriage in Plasmodium falciparum-infected travellers. *Malaria Journal*. 12(1):1–6. DOI: 10.1186/1475-2875-12-31.

Roberts, K.W., Gueye, C.S., Baltzell, K., Ntuku, H., Mccreesh, P., Maglior, A., Whittemore, B., Uusiku, P., et al. 2021. Community acceptance of reactive focal mass drug administration and reactive focal vector control using indoor residual spraying , a mixed - methods study in Zambezi region , Namibia. *Malaria Journal*. 1–11. DOI: 10.1186/s12936-021-03679-1.

Robinson, A., Busula, A.O., Muwanguzi, J.K., Powers, S.J., Masiga, D.K., Bousema, T., Takken, W., de Boer, J.G., et al. 2019. Molecular quantification of Plasmodium parasite density from the blood retained in used RDTs. *Scientific Reports*. 9(1):1–8. DOI: 10.1038/s41598-019-41438-0.

Russell, T.L., Govella, N.J., Azizi, S., Drakeley, C.J., Kachur, S.P. & Killeen, G.F. 2011. Increased proportions of outdoor feeding among residual malaria vector populations following increased use of insecticide-treated nets in rural Tanzania. *Malaria Journal*. 10:1–10. DOI: 10.1186/1475-2875-10-80.

Russell, T.L., Beebe, N.W., Cooper, R.D., Lobo, N.F. & Burkot, T.R. 2013. Successful malaria elimination strategies require interventions that target changing vector behaviours. *Malaria Journal*. 12(1):1–5. DOI: 10.1186/1475-2875-12-56.

Salgado, C., Ayodo, G., Macklin, M.D., Gould, M.P., Nallandhighal, S., Odhiambo, E.O., Obala, A., O'Meara, W.P., et al. 2021. The prevalence and density of asymptomatic Plasmodium falciparum infections among children and adults in three communities of western Kenya. *Malaria Journal*. 20(1):1–11. DOI: 10.1186/s12936-021-03905-w.

Sato, S. 2021. Plasmodium — a brief introduction to the parasites causing human malaria and their basic biology. 9:1–13.

Sattabongkot, J., Tsuboi, T., Han, E. & Bantuchai, S. 2014. Loop-Mediated

Isothermal Amplification Assay for Rapid Diagnosis of Malaria Infections in an Area of Endemicity in Thailand. 52(5):1471–1477. DOI: 10.1128/JCM.03313-13.

Schlabe, S., Reiter-Owona, I., Nordmann, T., Dolscheid-Pommerich, R., Tannich, E., Hoerauf, A. & Rockstroh, J. 2021. Rapid diagnostic test negative Plasmodium falciparum malaria in a traveller returning from Ethiopia. *Malaria Journal*. 20(1):1–7. DOI: 10.1186/s12936-021-03678-2.

Schneider, P., Reece, S.E., Van Schaijk, B.C.L., Bousema, T., Lanke, K.H.W., Meaden, C.S.J., Gadalla, A., Ranford-Cartwright, L.C., et al. 2015. Quantification of female and male Plasmodium falciparum gametocytes by reverse transcriptase quantitative PCR. *Molecular and Biochemical Parasitology*. 199(1–2):29–33. DOI: 10.1016/j.molbiopara.2015.03.006.

Searle, K.M., Hamapumbu, H., Lubinda, J., Shields, T.M., Pinchoff, J., Kobayashi, T., Stevenson, J.C., Bridges, D.J., et al. 2016. Evaluation of the operational challenges in implementing reactive screen - and - treat and implications of reactive case detection strategies for malaria elimination in a region of low transmission in southern Zambia. *Malaria Journal*. 1–14. DOI: 10.1186/s12936-016-1460-x.

Selvarajah, D., Naing, C., Htet, N.H. & Mak, J.W. 2020. Loop-mediated isothermal amplification (LAMP) test for diagnosis of uncomplicated malaria in endemic areas: A meta-analysis of diagnostic test accuracy. *Malaria Journal*. 19(1):1–10. DOI: 10.1186/s12936-020-03283-9.

Shankar, H., Singh, M.P., Phookan, S., Singh, K. & Mishra, N. 2021. Diagnostic performance of rapid diagnostic test , light microscopy and polymerase chain reaction during mass survey conducted in low and high malaria-endemic areas from two North-Eastern states of India. 2251–2261.

Sherrard-Smith, E., Skarp, J.E., Beale, A.D., Fornadel, C., Norris, L.C., Moore, S.J., Mihreteab, S., Charlwood, J.D., et al. 2019. Mosquito feeding behavior and how it influences residual malaria transmission across Africa. *Proceedings of the National Academy of Sciences*. 116(30):15086–15095. DOI: 10.1073/pnas.1820646116.

Shibeshi, M.A., Kifle, Z.D. & Atnafie, S.A. 2020. Antimalarial drug resistance and novel targets for antimalarial drug discovery. *Infection and Drug Resistance*. 13:4047–4060. DOI: 10.2147/IDR.S279433.

Siahaan, L. 2018. Laboratory diagnostics of malaria. *IOP Conference Series: Earth and Environmental Science*. 125(1). DOI: 10.1088/1755-1315/125/1/012090.

Singh, B. & Daneshvar, C. 2013. Human infections and detection of plasmodium knowlesi. *Clinical Microbiology Reviews*. 26(2):165–184. DOI: 10.1128/CMR.00079-12.

Singh, U.A., Kumari, M. & Iyengar, S. 2018. Method for improving the quality of genomic DNA obtained from minute quantities of tissue and blood samples using Chelex 100 resin. *Biological Procedures Online*. 20(1):1–8. DOI: 10.1186/s12575-018-0077-6.

Smith, D.M. 2010. Malaria Diagnosis by a Polymerase Chain Reaction-Based Assay Using a Pooling Strategy. 81(5):754–757. DOI: 10.4269/ajtmh.2009.09-0274.Malaria.

Smith, J.L., Auala, J., Tambo, M., Haindongo, E., Katokele, S., Uusiku, P., Gosling, R., Kleinschmidt, I., et al. 2017. Spatial clustering of patent and sub-patent malaria infections in northern Namibia : Implications for surveillance and response strategies for elimination. 1–13.

Smith, J.L., Auala, J., Haindongo, E., Uusiku, P., Gosling, R., Kleinschmidt, I., Mumbengegwi, D. & Sturrock, H.J.W. 2017. Malaria risk in young male travellers but local transmission persists: A case-control study in low transmission Namibia. *Malaria Journal*. 16(1):1–13. DOI: 10.1186/s12936-017-1719-x.

Smith, J.L., Mumbengegwi, D., Id, E.H., Cueto, C., Id, K.W.R., Gosling, R., Uusiku, P., Kleinschmidt, I., et al. 2021. Malaria risk factors in northern Namibia : The importance of occupation , age and mobility in characterizing high-risk populations. 1–23. DOI: 10.1371/journal.pone.0252690.

Sondo, P., Bihoun, B., Tahita, M.C., Derra, K., Diallo, S.N., Kazienga, A., Ilboudo, H., Valea, I., et al. 2021. Plasmodium falciparum gametocyte carriage in symptomatic patients shows significant association with genetically diverse infections , anaemia , and asexual stage density To cite this version : Plasmodium falciparum gametocyte carriage in symptomatic patie. *Malaria Journal*. DOI: 10.1186/s12936-020-03559-0.

Stepniewska, K., Humphreys, G.S., Gonçalves, B.P., Craig, E., Gosling, R., Guerin, P.J. & Price, R.N. 2022. Efficacy of Single-Dose Primaquine With Artemisinin Combination Therapy on Plasmodium falciparum Gametocytes and Transmission : An Individual Patient. 225:1215–1226. DOI: 10.1093/infdis/jiaa498.

Stresman, G., Kobayashi, T., Kamanga, A., Thuma, P.E., Mharakurwa, S., Moss, W.J. & Shiff, C. 2012. Malaria research challenges in low prevalence settings. *Malaria Journal*. 11:2–5. DOI: 10.1186/1475-2875-11-353.

Stresman, G.H., Kamanga, A., Moono, P., Hamapumbu, H., Mharakurwa, S., Kobayashi, T., Moss, W.J. & Shiff, C. 2010. A method of active case detection to target reservoirs of asymptomatic malaria and gametocyte carriers in a rural area in

Southern Province, Zambia. *Malaria Journal*. 9(1):1–8. DOI: 10.1186/1475-2875-9-265.

Strøm, G.E.A., Tellevik, M.G., Hanevik, K., Langeland, N. & Blomberg, B. 2014. Comparison of four methods for extracting DNA from dried blood on filter paper for PCR targeting the mitochondrial Plasmodium genome. *Transactions of the Royal Society of Tropical Medicine and Hygiene*. 108(8):488–494. DOI: 10.1093/trstmh/tru084.

Sturrock, H.J.W., Novotny, J.M., Kunene, S., Dlamini, S., Zulu, Z., Cohen, J.M., Hsiang, M.S., Greenhouse, B., et al. 2013. Reactive Case Detection for Malaria Elimination : Real-Life Experience from an Ongoing Program in Swaziland. 8(5). DOI: 10.1371/journal.pone.0063830.

Tadesse, F.G., Meerstein-kessel, L., Gonçalves, B.P., Drakeley, C., Ranford-cartwright, L. & Bousema, T. 2019. Gametocyte Sex Ratio : The Key to Understanding Plasmodium falciparum Transmission ? *Trends in Parasitology*. 35(3):226–238. DOI: 10.1016/j.pt.2018.12.001.

Tambo, M., Auala, J.R., Sturrock, H.J., Kleinschmidt, I., Bock, R., Smith, J.L., Gosling, R. & Mumbengegwi, D.R. 2018. Evaluation of loop-mediated isothermal amplification as a surveillance tool for malaria in reactive case detection moving towards elimination. *Malaria Journal*. 17(1):4–9. DOI: 10.1186/s12936-018-2399-x.

Tamiru, A., Tolossa, T. & Regasa, B. 2022. Prevalence of asymptomatic malaria and associated factors in Ethiopia : Systematic review and meta-analysis. DOI: 10.1177/20503121221088085.

Tangpukdee, N., Duangdee, C., Wilairatana, P. & Krudsood, S. 2009. Malaria diagnosis: A brief review. *Korean Journal of Parasitology*. 47(2):93–102. DOI:

10.3347/kjp.2009.47.2.93.

Tani, H., Tada, Y., Sasai, K. & Baba, E. 2008. Improvement of DNA extraction method for dried blood spots and comparison of four PCR methods for detection of Babesia gibsoni (Asian genotype) infection in canine blood samples. *Journal of Veterinary Medical Science*. 70(5):461–467. DOI: 10.1292/jvms.70.461.

Tankeshwar, A. 2022. Microbe Online. Thick and Thin Blood Smear for Malaria Diagnosis. https://microbeonline.com/microscopic-diagnosis-of-malaria/ Retrieved on: 14 August 2022. 1–11.

Tedla, M. 2019. A focus on improving molecular diagnostic approaches to malaria control and elimination in low transmission settings : Review. *Parasite Epidemiology and Control*. 6:e00107. DOI: 10.1016/j.parepi.2019.e00107.

Tesfahunegn, A., Berhe, G. & Gebregziabher, E. 2019. Risk factors associated with malaria outbreak in Laelay Adyabo district northern Ethiopia , 2017 : case-control study design. 1–7.

Tessema, S., Wesolowski, A., Chen, A., Murphy, M., Wilheim, J., Mupiri, A.R., Ruktanonchai, N.W., Alegana, V.A., et al. 2019. Using parasite genetic and human mobility data to infer local and cross- border malaria connectivity in Southern Africa. *eLife*. 8:1–20. DOI: 10.7554/eLife.43510.

Thompson, T.A., Touré, M.B., Sanogo, D., Shaffer, J.G., Doumbia, S.O. & Krogstad, D.J. 2020. Template copy number and the sensitivity of quantitative PCR for Plasmodium falciparum in asymptomatic individuals. *Malaria Journal*. 19(1):1–12. DOI: 10.1186/s12936-020-03365-8.

Tizifa, T.A., Kabaghe, A.N., McCann, R.S., van den Berg, H., Van Vugt, M. &

Phiri, K.S. 2018. Prevention Efforts for Malaria. *Current Tropical Medicine Reports*. 5(1):41–50. DOI: 10.1007/s40475-018-0133-y.

Ugah, U.I., Alo, M.N., Owolabi, J.O., Divinegift, O., Nwali, O., Ekejindu, I.M., Ibeh, N. & Elom, M.O. 2017. Evaluation of the utility value of three diagnostic methods in the detection of malaria parasites in endemic area. *Malaria Journal*. 1–8. DOI: 10.1186/s12936-017-1838-4.

Unterborn, R., Henao-cordero, J., Kousari, A., Ramanan, P., Franco-paredes, C. & Madinger, N. 2022. False-positive rapid diagnostic test for malaria in new world cutaneous leishmaniasis : a tale of two travelers. (X):4–7. DOI: 10.1177/20499361221097791.

Vandermosten, L., Pham, T.T., Possemiers, H., Knoops, S., Van Herck, E., Deckers, J., Franke-Fayard, B., Lamb, T.J., et al. 2018. Experimental malaria-associated acute respiratory distress syndrome is dependent on the parasite-host combination and coincides with normocyte invasion. *Malaria Journal*. 17(1):1–17. DOI: 10.1186/s12936-018-2251-3.

Venugopal, K., Hentzschel, F., Valkiūnas, G. & Marti, M. 2020. Plasmodium asexual growth and sexual development in the haematopoietic niche of the host. *Nature Reviews Microbiology*. 18(3):177–189. DOI: 10.1038/s41579-019-0306-2.

Wampfler, R., Mwingira, F., Javati, S., Robinson, L., Betuela, I., Siba, P., Beck, H.P., Mueller, I., et al. 2013. Strategies for Detection of Plasmodium species Gametocytes. *PLoS ONE*. 8(9):6–9. DOI: 10.1371/journal.pone.0076316.

Weiss, D.J., Bertozzi-Villa, A., Rumisha, S.F., Amratia, P., Arambepola, R., Battle, K.E., Cameron, E., Chestnutt, E., et al. 2021. Indirect effects of the COVID-19 pandemic on malaria intervention coverage, morbidity, and mortality in Africa: a

geospatial modelling analysis. *The Lancet Infectious Diseases*. 21(1):59–69. DOI: 10.1016/S1473-3099(20)30700-3.

Winskill, P., Walker, P.G., Cibulskis, R.E. & Ghani, A.C. 2020. Prioritizing the scale - up of interventions for malaria control and elimination. *Malaria Journal*. (2019):1–11. DOI: 10.1186/s12936-019-2755-5.

Wirth, D.F., Casamitjana, N., Tanner, M. & Reich, M.R. 2018. Global action for training in malaria elimination. *Malaria Journal*. 4–7. DOI: 10.1186/s12936-018-2199-3.

World Health Organization. 2008. WORLD HEALTH ORGANIZATION. GLOBAL MALARIA CONTROL AND ELIMINATION: report of a technical review.

World Health Organization. 2015. World Health Organization. Guidelines For the treatment of malaria.

World Health Organization. 2020. *World Health Organization 2020*. V. 73. Available: https://www.who.int/teams/global-malaria-programme/reports/world-malaria-report-2020.

World Health Organization. 2022. WHO Guidelines for malaria - 3 June 2022.

World Health Organization, 2012. 2012. Disease Surveillance for Malaria Control: An operational manual.

World Health Organization, 2021. 2021. *World Health Organization, 2021*. V. 13.

Yin, J., Yan, H. & Li, M. 2022. Prompt and precise identification of various sources of infection in response to the prevention of malaria re-establishment in China. *Infectious Diseases of Poverty*. 11(1):4–9. DOI: 10.1186/s40249-022-00968-y.

Zavala, F. 2022. RTS , S : the first malaria vaccine. 1–3.

Zemene, E., Koepfli, C., Tiruneh, A., Yeshiwondim, A.K., Seyoum, D., Lee, M.C., Yan, G. & Yewhalaw, D. 2018. Detection of foci of residual malaria transmission through reactive case detection in Ethiopia. *Malaria Journal*. 1–10. DOI: 10.1186/s12936-018-2537-5.

Appendix 1: Ethical Clearance Certificate

UNAM
UNIVERSITY OF NAMIBIA

ETHICAL CLEARANCE CERTIFICATE

Ethical Clearance Reference Number: SOS-0029 Date: 04 March 2022

This Ethical Clearance Certificate is issued by the University of Namibia Ethics Committee (REC) in accordance with the University of Namibia's Research Ethics Policy and Guidelines. Ethical approval is given in respect of undertakings contained in the Research Project outlined below. This Certificate is issued on the recommendations of the ethical evaluation done by the ethics committee.

Title of Project: DETERMINATION OF THE PREVALENCE AND DENSITY OF TRUE *PLASMODIUM FALCIPARUM* INFECTIONS AND GAMETOCYTE CARRIAGE DURING TWO MALARIA OUTBREAKS IN ZAMBEZI REGION, NAMIBIA

Student: LYDIA ELOFF

Student Number: 201603185

Supervisor(s): Prof. Davis Mumbengegwi

Centre for Research Services

Take note of the following:

1. Any significant changes in the conditions or undertakings outlined in the approved Proposal must be communicated to the ethics committee. An application to make amendments may be necessary.
2. Any breaches of ethical undertakings or practices that have an impact on ethical conduct of the research must be reported to the ethics committee
3. The Principal Researcher must report issues of ethical compliance to the ethics committee (through the Chairperson) at the end of the Project or as may be requested by the ethics committee
4. The ethics committee retains the right to:

i) Withdraw or amend this Ethical Clearance if any unethical practices (as outlined in the Research Ethics Policy) have been detected or suspected,

ii) Request for an ethical compliance report at any point during the course of the research.

The ethics committee wishes you the best in your research.

Dr. Zivayi Chiguvare (Chairperson Ethics Committee)

p.p.

Prof. Davis Mumbengegwi (Head, Multidisciplinary Research)

Appendix 2: Statistical analysis for 2017 and 2021 parasitaemia and gametocytaemia.

Tests of Normality

values 1 or 7		Kolmogorov-Smirnov[a] Statistic	df	Sig.	Shapiro-Wilk Statistic	df	Sig.
2021	Outbreak 2017 and 2021 Parasite density	.404	152	<.001	.204	152	<.001
2017	Outbreak 2017 and 2021 Parasite density	.305	71	<.001	.545	71	<.001

a. Lilliefors Significance Correction

NPar Tests

Mann-Whitney Test

Ranks

	values 1 or 7	N	Mean Rank	Sum of Ranks
Outbreak 2017 and 2021 Parasite density	2021	152	104.13	15828.00
	2017	71	128.85	9148.00
	Total	223		

Test Statistics[a]

	Outbreak 2017 and 2021 Parasite density
Mann-Whitney U	4200.000
Wilcoxon W	15828.000
Z	-2.665
Asymp. Sig. (2-tailed)	.008

a. Grouping Variable: values 1 or 7

	values 1 or 7			Statistic	Std. Error
Outbreak 2017 and 2021 Parasite density	2021	Mean		35210.9294	11788.17001
		95% Confidence Interval for Mean	Lower Bound	11919.8758	
			Upper Bound	58501.9831	
		5% Trimmed Mean		16680.6100	
		Median		4442.9837	
		Variance		2.112E+10	
		Std. Deviation		145334.3205	
		Minimum		.70	
		Maximum		1.70E+6	
		Range		1702034.12	
		Interquartile Range		26765.57	
		Skewness		10.290	.197
		Kurtosis		116.589	.391
	2017	Mean		46088.4427	10414.31971
		95% Confidence Interval for Mean	Lower Bound	25317.7369	
			Upper Bound	66859.1484	
		5% Trimmed Mean		30312.1904	
		Median		17436.4813	
		Variance		7700521906	
		Std. Deviation		87752.61766	
		Minimum		2.25	
		Maximum		455153.08	
		Range		455150.83	
		Interquartile Range		36413.80	
		Skewness		3.293	.285
		Kurtosis		11.534	.563

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	values 1 and 7	Statistic	df	Sig.	Statistic	df	Sig.
Outbreak 2017 and 2021 Gametocytaemia	2021	.345	98	<.001	.437	98	<.001
	2017	.331	59	<.001	.450	59	<.001

a. Lilliefors Significance Correction

Mann-Whitney Test

Ranks

	Values 1 for 2021 and 7 for 2017	N	Mean Rank	Sum of Ranks
Outbreak 2017 and 2021 Gametocyte Density	2021	98	87.28	8553.00
	2017	59	65.25	3850.00
	Total	157		

Test Statistics[a]

	Outbreak 2017 and 2021 Gametocyte Density
Mann-Whitney U	2080.000
Wilcoxon W	3850.000
Z	-2.939
Asymp. Sig. (2-tailed)	.003

a. Grouping Variable: Values 1 for 2021 and 7 for 2017

	values 1 and 7			Statistic	Std. Error
Outbreak 2017 and 2021 Gametocytaemia	2021	Mean		892.2333	224.96017
		95% Confidence Interval for Mean	Lower Bound	445.7496	
			Upper Bound	1338.7170	
		5% Trimmed Mean		473.9936	
		Median		99.9884	
		Variance		4959493.860	
		Std. Deviation		2226.99211	
		Minimum		4.52	
		Maximum		13335.21	
		Range		13330.70	
		Interquartile Range		662.82	
		Skewness		3.958	.244
		Kurtosis		16.880	.483
	2017	Mean		115.9909	33.30818
		95% Confidence Interval for Mean	Lower Bound	49.3173	
			Upper Bound	182.6644	
		5% Trimmed Mean		70.4797	
		Median		34.0987	
		Variance		65456.641	
		Std. Deviation		255.84495	
		Minimum		4.45	
		Maximum		1671.92	
		Range		1667.46	
		Interquartile Range		87.03	
		Skewness		4.535	.311

Nonparametric Correlations

[DataSet0]

Correlations

			Parasitaemia 2017	Gametocytaemia2017
Spearman's rho	Parasitaemia 2017	Correlation Coefficient	1.000	.600
		Sig. (2-tailed)	.	.400
		N	4	4
	Gametocytaemia2017	Correlation Coefficient	.600	1.000
		Sig. (2-tailed)	.400	.
		N	4	4

Nonparametric Correlations

Correlations

			Parasitaemia 2021	Gametocytaemia 2021
Spearman's rho	Parasitaemia 2021	Correlation Coefficient	1.000	1.000**
		Sig. (2-tailed)	.	.
		N	4	4
	Gametocytaemia 2021	Correlation Coefficient	1.000**	1.000
		Sig. (2-tailed)	.	.
		N	4	4

**. Correlation is significant at the 0.01 level (2-tailed).

NPar Tests

Median Test

Frequencies

		Categorised Parasitaemia 2021			
		Low	Moderate	High	Hyperparasitaemia
Gametocytaemia 2021	> Median	5	12	24	8
	<= Median	9	15	23	2

Test Statistics[a]

	Gametocytaemia 2021
N	98
Median	99.9884
Chi-Square	5.097[b]
df	3
Asymp. Sig.	.165

a. Grouping Variable: Categorised Parasitaemia 2021

b. 0 cells (0.0%) have expected frequencies

NPar Tests

Median Test

Frequencies

		Categorised Parasitaemia 2021	
		Low	Moderate
Gametocytaemia 2021	> Median	7	13
	<= Median	7	14

Test Statistics[a]

		Gametocytaemia 2021
N		41
Median		31.6228
Chi-Square		.013
df		1
Asymp. Sig.		.910
Yates' Continuity Correction	Chi-Square	.047
	df	1
	Asymp. Sig.	.828

a. Grouping Variable: Categorised Parasitaemia 2021

NPar Tests

Warnings

There are not enough valid cases to perform the Median Test for Gametocytaemia 2017 * Parasitaemia 2017 (11.00, 12.00). No statistics are computed.

NPar Tests

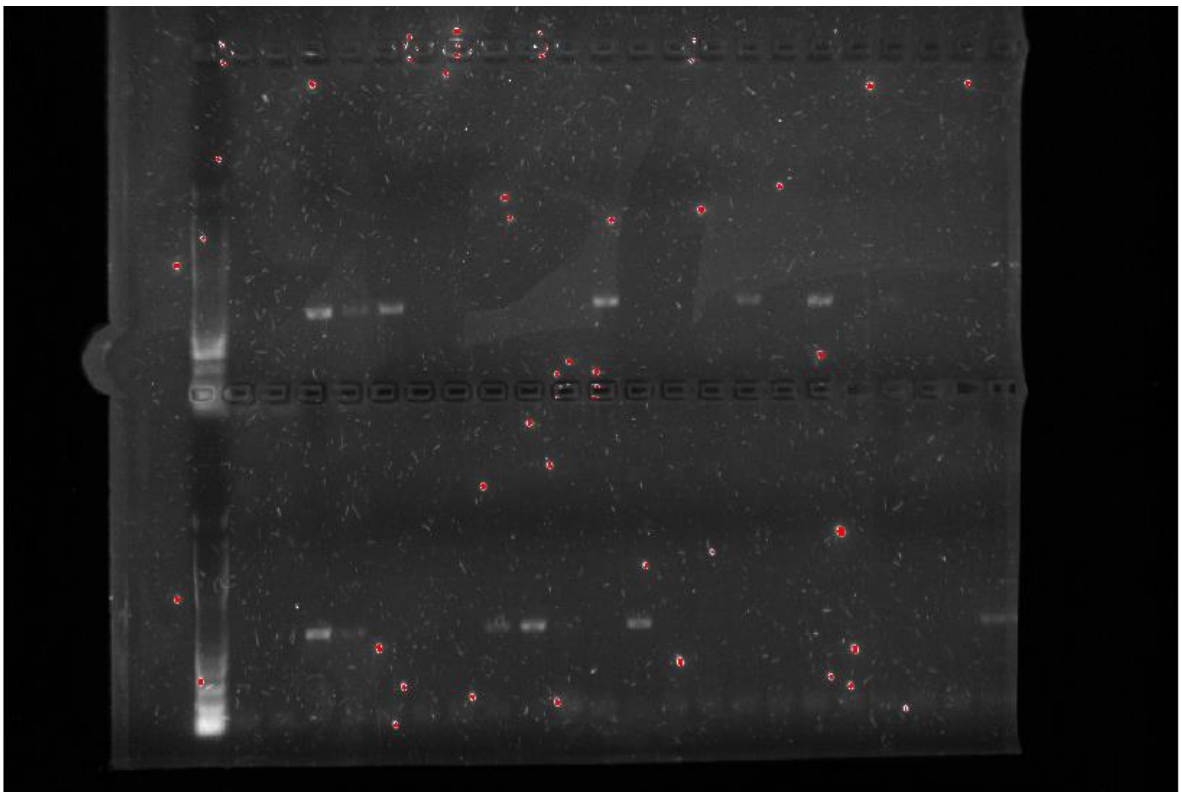
Warnings

There are not enough valid cases to perform the Median Test for Gametocytaemia 2017 * Parasitaemia 2017 (11.00, 14.00). No statistics are computed.

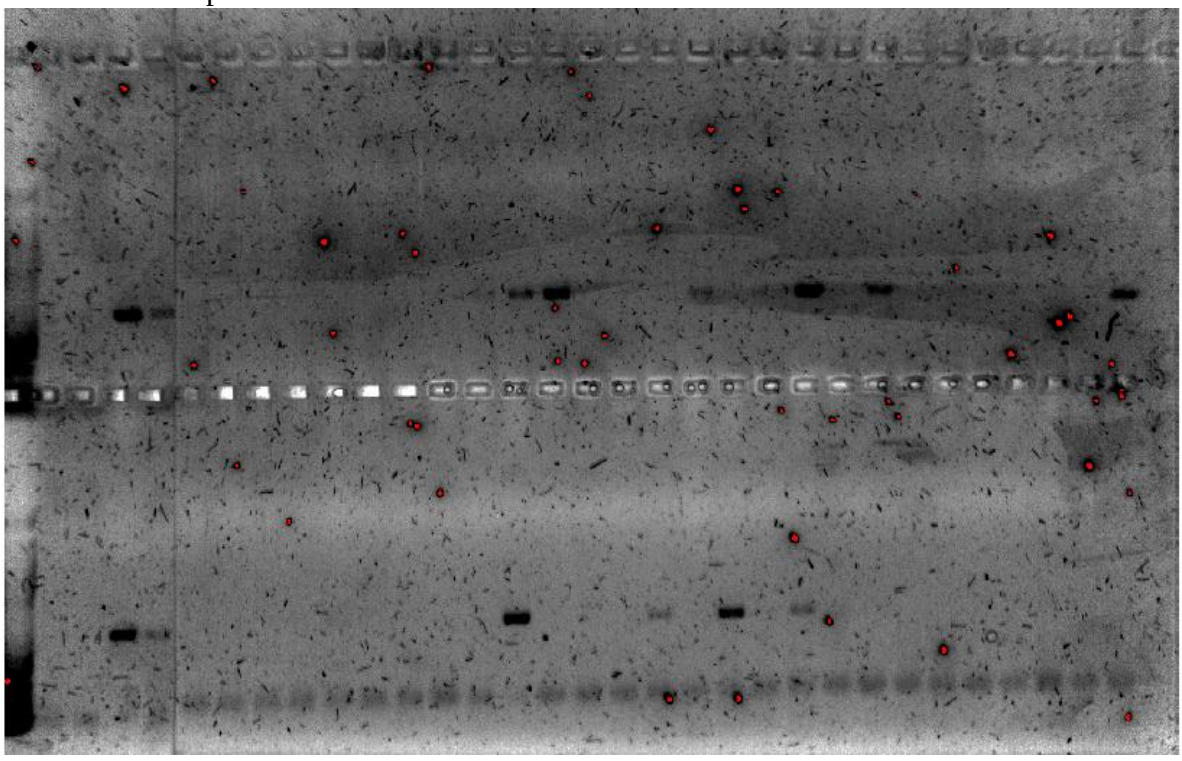
Appendix 3: Gel Results
Malaria Outbreak 2017 PCR Results

First Run

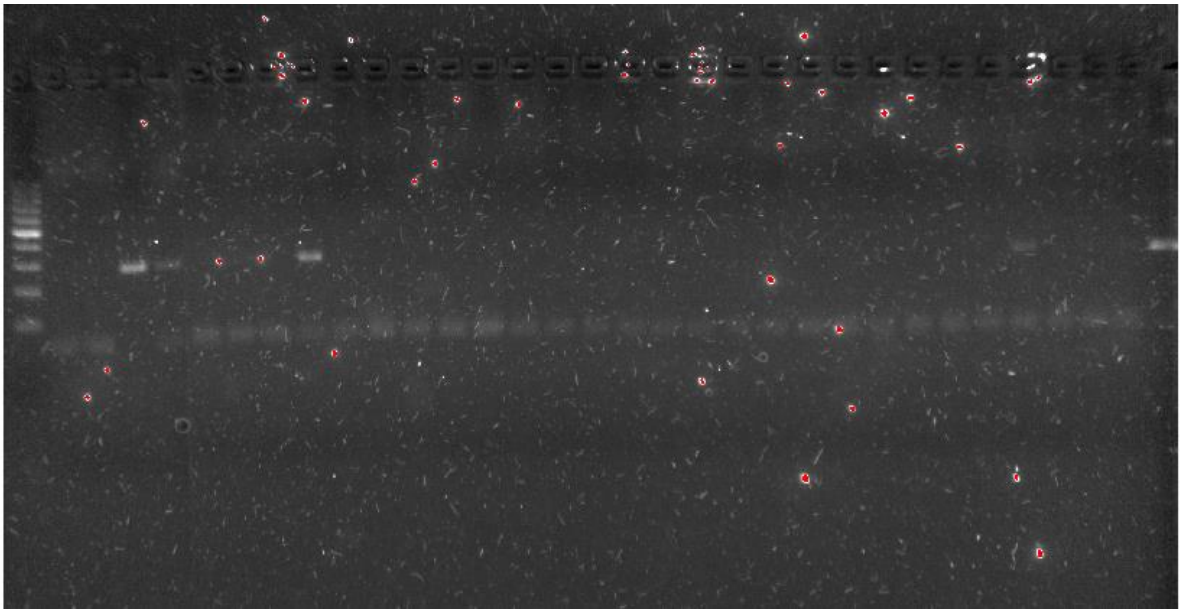
Outbreak2017 top1-18bottom47-64



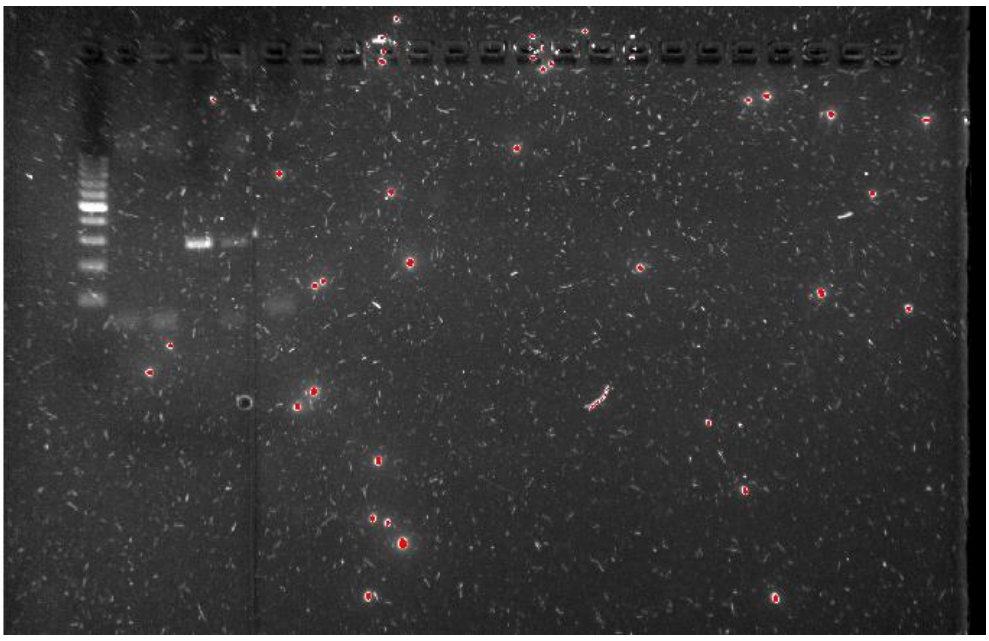
Outbreak2017 top19-46bottom65-91



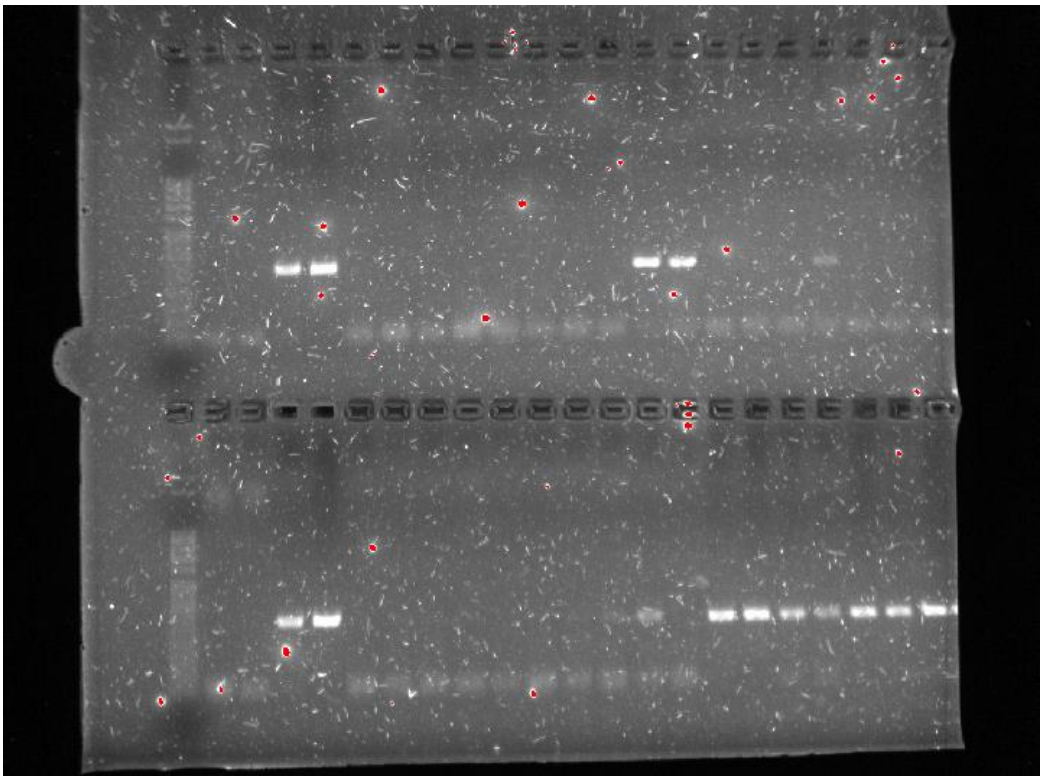
Outbreak2017 top92-119



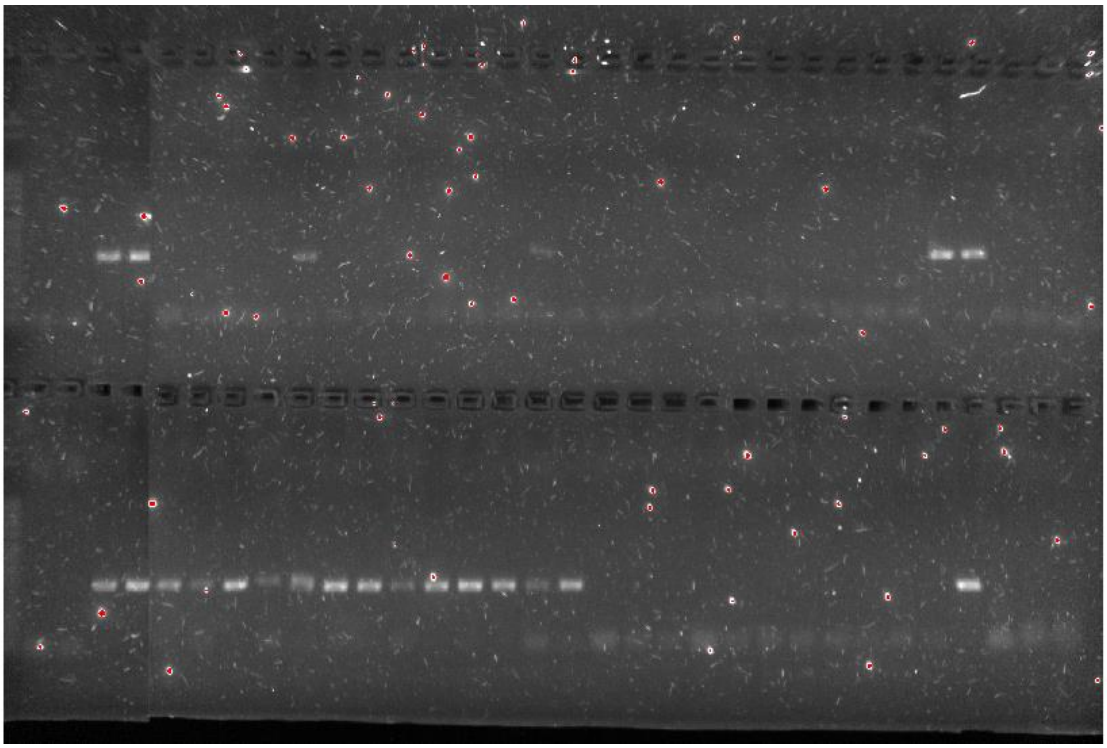
Outbreak2017 top120



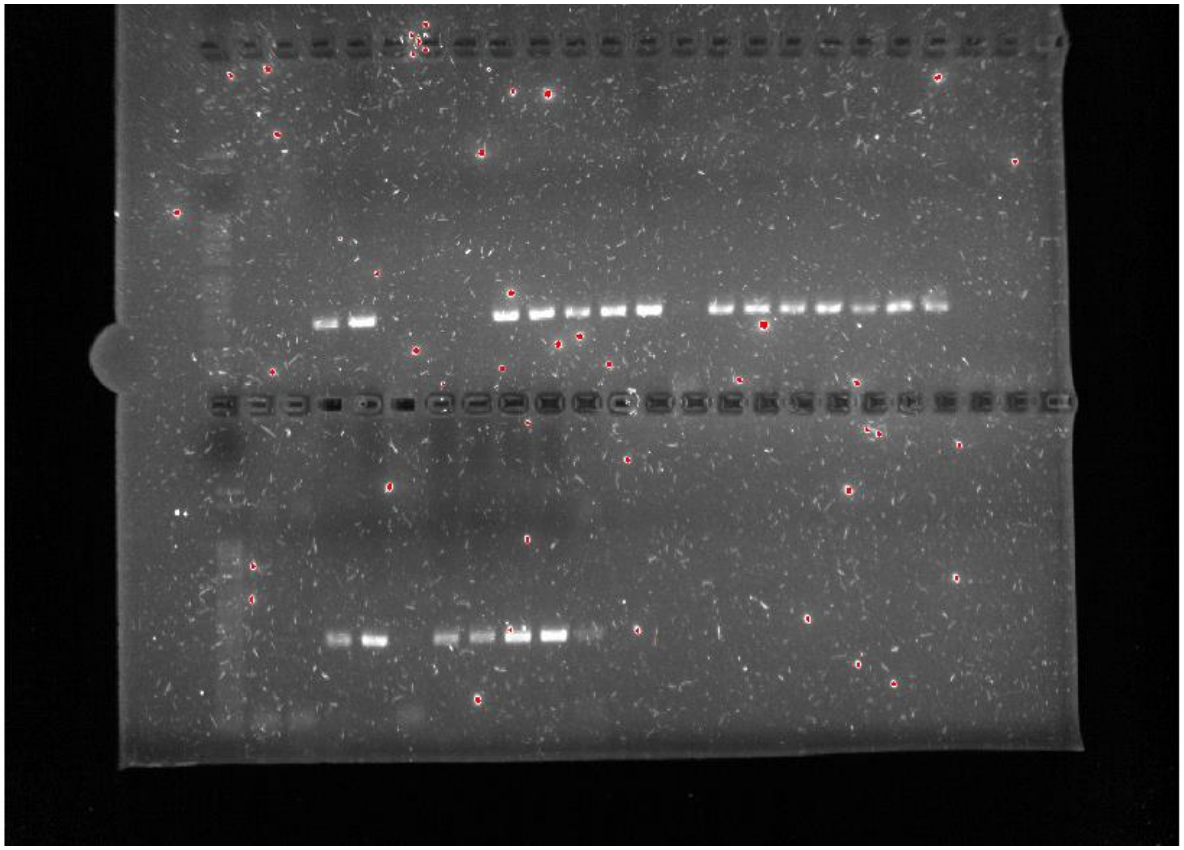
Outbreak2017 top121-137bottom167-183



Outbreak2017 top138-166bottom184-211



Outbreak2017 top212-230bottom



Outbreak2017 top231-251 possible mixed infection in lane22