MALARIA IN NATA VILLAGE, NORTHERN BOTSWANA: EVALUATION OF VECTOR CONTROL METHODS

By

ODUETSE IVAN NKAIWA

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Main Supervisor: Prof. M.M.O. Thekisoe

Co-supervisor: Dr S.J. Nkhebenyane

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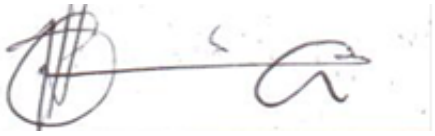
DECLARATION OF INDEPENDENT WORK

I, the undersigned, do hereby declare that this research project submitted to the **Central University of Technology, Free State**, for the degree **MASTER OF HEALTH SCIENCES IN ENVIRONMENTAL HEALTH** is my own original and independent research work that is true and authentic. This research work has not been submitted before to any institution by me or any other person in fulfilment of the requirements for the attainment of any qualification.



08 October 2019

Oduetse Ivan Nkaiwa **Date**

DEDICATIONS

I dedicate this dissertation to my parents, Mr Godfrey Nkaiwa and Mrs Rosinah Nkaiwa. Mumzo and dad, I thank you for your continuous support from the day I decided to further my studies. To my bestie, Kgomotso Mothibi, thank you for your dedication and encouragement.

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LIST OF ABBREVIATIONS

ACT- Artemisinin-based Combination Therapy

AIDS- Acquired Immune Deficiency Syndrome

AL- Arthemether-lumenfantrine

AS/AQ- Artesunate-amodiaquine

BOPA- Botswana Press Agency

C- Carbamates

CDC- Centre for Disease Control and Prevention

COI/ CO1- Cytochrome c oxidase I

CS- Capsule suspension

CQ- Chloroquine

DDE- Dichlorodiphenyltrichloroethylene

DDT- Dichlorodiphenyltrichloroethane

DNA- Deoxyribonucleic Acid

EC- Emulsifiable concentrate

GFATM- Global Fund to Fight AIDS, Tuberculosis and Malaria

IRS- Indoor Residual Spraying

ITNs- Insecticide Treated Nets

IVM- Integrated Vector Management

LLINs- Long-lasting Insecticide Treated Nets

MCL- Maximum Composite Likelihood

ML- Maximum Likelihood

MoH- Ministry of Health

NMCP- National Malaria Control Programme

NCBI- National Centre for Biotechnology Information

OC- Organochlorine

OP- Organophosphate

PCR- Polymerase Chain Reaction

PY- Pyrethroid

RDT- Rapid Diagnostic Test

RNA- Ribonucleic acid

rRNA- ribosomal RNA

SADC- Southern African Development Community

SC- Suspension concentrate

SR- Source Reduction

USA- United States of America

WG- Water dispersible granule

WHO- World Health Organization

WHO AFRO- World Health Organization African Region

WP- Wettable powder

SUMMARY

The main aim of this study was three-fold: to determine villagers' knowledge about preferences for malaria control interventions; to investigate which mosquito species were prevalent in Nata village, and to determine whether the *Plasmodium* parasite that is carried by mosquitoes existed in Nata village from the sampled mosquitoes. Nata village is located in the Tutume malaria-endemic sub-district in Botswana. To meet the first aim of the study, interviewer-administered questionnaires which were completed by 109 volunteer participants. To address the second and third aims, Mosquito specimens were collected by using the H-trap and spray sheet collection methods. For the identification of mosquito species, morphological characteristics and molecular analysis techniques were used. Basic taxonomic keys (wings, abdomen, legs, and proboscis) were examined for morphological identification while the polymerase chain reaction (PCR) was mainly utilized for the molecular identification and detection of the *Plasmodium* parasite in the collected mosquito samples.

A total of 109 participants consented to take part in the study. The majority (65%) of the participants were females and all the participants (100%) claimed to know about malaria control interventions. Only a small group (12%) of these study participants preferred the use of both long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) for sustained use and protection against malaria. Of the 27 mosquitoes used for the morphological analyses, 41% was identified as the *Anopheles* species while 59% was identified as *Culex*. Through PCR and the use of species specific primer, no *Plasmodium* parasites were detected in the DNA of any of the mosquitoes. It was thus

established that the mosquitoes in the study area were not infected with the malaria parasite at the time of the study. However, the presence of the possible potential carriers of this parasite in this area suggests that the threat of malaria may still exist.

For molecular identification of the mosquito sample, PCR was conducted with primers targeting CO1, 16S rRNA, and 18S rRNA. Only 16S rRNA was used for further analysis because no significant matches could be found on the NCBI database for CO1 and 18S rRNA sequences. Through phylogenetic analyses, three genera of mosquitoes, namely *Anopheles*, *Aedes* and *Culex*, were identified as prevalent in the study area. Seven mosquito species, including *Culex quinquefasciatus*, *Culex pipiens quinquefasciatus*, *Culex coronator*, *Aedes aegypti*, *Aedes arborealis*, *Anopheles maculatus*, and *Anopheles eiseni* were identified through phylogenetic analysis that revealed the existence of multiple mosquito species in Nata village.

PCR analysis of the samples revealed the presence of vectors of diseases other than malaria. This investigation also elicited knowledge regarding the preferred methods of malaria control initiatives used by the community. This knowledge should be disseminated to support the implementation of environmentally safe malaria control tools and thereby improving the quality of life and health of humans in malaria-prone regions. This study revealed new findings about hitherto unsuspected species of mosquitoes that might co-exist with malaria carrier species or that might even influence malaria distribution in the study area. This is the first report that identified *Aedes* and *Culex* genera in Nata village or in the district where the village is situated, and the

finding thus calls for further studies to determine the actual role played by these vectors in a malaria endemic area.

CHAPTER 1

INTRODUCTION AND BACKGROUND

TO THE STUDY

1.1 General Introduction

Malaria is a life threatening parasitic disease transmitted by female *Anopheles* mosquitoes. It is also the most threatening parasitic disease that impacts the health of human beings. It is transmitted by mosquitoes in 108 countries that are inhabited by roughly 3 billion people. In 2010, malaria caused an estimated 216 million cases of infection and 655 000 deaths (White *et al*., 2014).

There are four types of *Plasmodium* species that infect humans, namely *Plasmodium vivax*, *P. malariae*, *P. ovale*, and *P. falciparum* (Akande and Musa, 2005; White *et* al., 2014; Greenwood *et al*., 2005). The *P. falciparum* and *P. vivax* are the most common of these species. It has been reported that *P. falciparum* causes the most deadly type of infection and that it occur most commonly in sub-Saharan Africa (White *et al*., 2014; Greenwood *et al*., 2005). A variety of environmental factors affect the distribution, seasonality and transmission intensity of malaria (Akande and Musa, 2005; Fana *et al*., 2015; White *et al*., 2014). Study by Aslan *et al*. (2007) further state that *P. falciparum* is responsible for the highest mortality rates and *P. vivax* causes considerable morbidity, while *P. malariae* and *P. ovale* are less prevalent around the world.

Rainfall provides breeding sites for mosquitoes and increases humidity which enhances their survival. In areas of stable malaria transmission, young children and pregnant women are most at risk for malaria morbidity and mortality. Additionally, population groups most at risk of epidemic outbreaks are those living in high lying areas, in arid

and desert fringe zones, and in areas where successful control measures have not been consolidated or maintained.

1.2 Overview of Malaria in Botswana

Botswana is a landlocked country with a total population of 2 024 904. It shares borders with Namibia, Zambia, Zimbabwe and South Africa (Chirebvu *et al*., 2016). The country comprises of land at 1000 meters above sea level, with substantial tracts of desert such as the well-known Kalahari in the south western region. Temperatures in the summer are above 35°C, while winter minimum temperatures can drop to 0°C and below, with occasional frost at night in the months of June to August. Botswana sometimes experiences high inter-annual periodic drought even though 90% of the rainfall occurs mainly during the summer months of October to April (Thomson *et al*., 2005; Chirebvu *et al*., 2016). The interplay of factors such as climate variability, socio-cultural differences and institutional diversity has seriously impeded malaria elimination efforts in Botswana; thus the disease remains a developmental problem that challenges livelihood sustainability and the effectiveness of health systems (Magole *et al*., 2017).

The *Anopheles arabiensis* species is the most widespread vector of *P. falciparum* in Botswana, even though some members of the *An. gambiae* and *An. funestus* groups have also been reported to be prevalent (Moakofhi *et al*., 2018; Obopile *et al*., 2018). It is stated by Motshoge *et al*. (2016) that 98% of malaria infections in Botswana are caused by *P. falciparum*, while 2% is attributed to *P. vivax.*

In 2016, Botswana reported 716 indigenous and 64 imported malaria cases; however, after thorough scrutiny of reports and the use of the private sector for malaria treatment,

WHO estimated that the final tally of cases of indigenous malaria was 1 911 at the time, which was much higher than originally reported. Compared with the 2015 figures of a total of 326 indigenous cases and 877 imported cases, the 2016 figures are an undeniable indication of an increasing trend in malaria transmission in Botswana. A recent stratification map shows a large number of cases along the Botswana and South African border in the Limpopo Province and along its northern and north-eastern borders with Namibia and Zimbabwe (WHO, 2018). It is reported by WHO (2018) that the National Malaria Programme under Botswana's Ministry of Health and Wellness had been fully funded by the government until 2015, and that a US$5.2 million Global Fund to fight AIDS, Tuberculosis and Malaria (GFATM) grant was received to support the elimination of malaria and other diseases until September 2018. However, the programme faced challenges related to the quality of case investigations due to low adherence to surveillance guidelines, poor uptake of vector control interventions by communities, and inadequate human resource capacity at all levels. Malaria transmission, as well as mosquito breeding, depends on rainfall and temperature. The northern part of Botswana experiences relatively high rainfall in the summer and mild temperatures in the winter, whereas the southern part has low rainfall in summer with mild temperatures (Motshoge *et al*., 2016). Therefore, due to the suitable climatic conditions for mosquito breeding and survival in some regions, malaria is endemic with frequent outbreaks. Malaria transmission trends in Botswana are seasonal and unstable, ranging from high endemicity in the northern part of the country to very low rates in the southern part of the country. However, all regions have had significant

malaria hotspots nation-wide for several consecutive years (Magole *et al*., 2017; Motshoge *et al*., 2016).

1.3 Malaria Control Strategies in Botswana

Malaria is ranked as a major public health problem in Botswana despite a National Malaria Control Programme (NMCP) dating back to the 1970s (MacLeod *et al*., 2015). The NMCP unit exists under the Department of Public Health in the Botswana's Ministry of Health and Wellness and its antimalarial activities are funded by the Global Fund and the Botswana government. The NMCP is responsible for the overall coordination of malaria surveillance, monitoring and evaluation, advocacy and information, education and communication, case management, and vector control (Motlaleng *et al*., 2018). In 2004, the World Health Organization African region (WHO AFRO) adopted integrated vector management (IVM) as a key malaria strategy for all affected countries, including Botswana. IVM advocates the use of different vector control methods in combination to reduce human-vector contact. These methods include long-lasting insecticide-treated nets (LLINs) and indoor residual spraying (IRS), which are currently the most successful malaria vector control interventions (Mpofu *et al*., 2016; Motlaleng *et al*., 2018), particularly in rural areas where malaria outbreaks may be rife.

The LLINs and IRS are also major methods for malaria control and prevention in Africa. Between 2014 and 2016, ~505 million LLINs were delivered to sub-Saharan countries, including Botswana. The free distribution policy of LLINs was adopted in 2009 in Botswana and the use of LLINs has been increasing in most countries (Wang, 2018). When optimally implemented, IRS and LLINs significantly reduce malaria parasite

prevalence in low-transmission settings. The LLINs protect the occupants of homes by diverting host seeking vectors and by killing those that attempt to feed. However, the number of these nets distributed annually since 2005 has remained below the required rate to reach universal access (Mpofu *et al*., 2016; Obopile *et al*., 2018). According to a biannual report on Botswana for 2016 and 2017 (WHO, 2018), IRS and LLINs coverage in Botswana fell short of the target set by WHO guidelines of 100% and 90% respectively. This suggests that more work still needs to be done to achieve the goal of a malaria-free Botswana.

The IRS coverage rates are calculated as the number of rooms sprayed in each house, yet the NMCP only targets living rooms for spraying and distributes LLINs door-to-door based on sleeping spaces or the distribution of one net for every two people in cases where sleeping spaces are not defined. The programme is also responsible for the demonstration of how the nets should be used, as well as malaria education. The LLINs are also distributed to children under the age of five through child welfare clinics and to pregnant women through antenatal clinics (Simon *et al*., 2013). The major challenge for IRS and LLINs is the ability of mosquitoes to develop resistance to the insecticides used in these interventions (Obopile *et al*., 2018). Therefore, larviciding has been recommended as a complementary intervention to IRS and LLINs and to be utilized in areas with few water bodies, fixed and easy to access (Mpofu *et al*., 2016).

1.4 Malaria Detection, Treatment and Management in Botswana

Malaria as a major health problem in Botswana is classified as a notifiable disease. Adequate case management reduces the negative effects of malaria infections for those who fall ill and it can also prevent transmission of the malaria parasite to new hosts by reducing the number of people with active malaria infections (Stelmach, 2013). Malaria may present clinically as either uncomplicated malaria, severe malaria, or remain as an asymptomatic infection, especially in people living in malaria endemic areas. In Botswana, due to the high seasonal and epidemic nature of malaria transmission, infection with malaria invariably leads to the clinical manifestation of the disease (Botswana Ministry of Health, 2007).

Insecticide Treated Nets (ITNs) and IRS are the first-line vector control tools for malaria. Antimalarial prophylaxis can be used for malaria prevention, especially for travellers. Microscopy or the rapid diagnostic test (RDT) is recommended for diagnostic testing of suspected malaria cases. Artemisinin-based combination therapy (ACT) is regarded as the best available therapy for *P. falciparum* malaria and chloroquine (CQ) is widely used for treating *P. vivax* (Wang, 2018). However, a report by Moakofhi *et al*. (2018) states that all countries in the African region have adopted either artesunate-amodiaquine (AS/AQ) or arthemether-lumenfantrine (AL) as their first-line treatment policy in line with WHO guidelines.

The treatment of malaria, and also its control interventions, is fraught with many challenges as the *P. falciparum* is often resistant to therapy (ACT). This fact prompted WHO to recommend in 2010 that all patients with suspected malaria should undergo serum microscopy or malaria rapid diagnostic testing (RDT), and that only those with a positive result should be treated (Moakofhi *et al*., 2018).

1.5 Rationale for the Study

Malaria is known to be caused by four *Plasmodium* species, namely *P. falciparum*, *P. ovale*, *P. vivax* and *P. malariae*, and these species are transmitted by the *Anopheles* mosquito (Akande and Musa, 2015). Treatment regimens have been successful in assisting people who contract malaria, but limited research has been done on the effectiveness of malaria control methods, the species of circulating vector mosquitoes, and the *Plasmodium* species that are harbouring this parasite in Nata village, Botswana. Furthermore, the perspectives of residents on malaria control methods have not been documented. This study thus endeavoured to address these gaps in scholarly knowledge.

As of March 2017, there were 843 confirmed cases of malaria and 8 deaths recorded in Botswana (Ministry of Health and Wellness, 2017). Although new malaria cases had dropped by 21% globally between 2010 and 2015, an estimated 43% of people in sub-Saharan Africa were not protected (WHO, 2017). Furthermore, (WHO, 2017) stated that, in the sub-Saharan African region, insecticide-treated nets had the greatest impact, accounting for an estimated 69% of cases prevented through controls. Global efforts in the last 15 years have led to a 62% reduction in malaria deaths between 2000 and

2015, yet approximately 429 000 people died of the disease in 2015, the majority being young children in Africa (WHO, 2017).

It is therefore envisaged that the findings of this study, when appropriately disseminated, will help to assist decision makers to understand the need to incorporate malaria elimination interventions into social-cultural dimensions of the affected community, thus encouraging community participation in malaria elimination strategies. This study has seemed to report on the authentic views of Nata residents regarding their preferred methods of malaria elimination and their perceptions on the effectiveness of these methods. Community involvement in the fight against malaria is vital, particularly in Botswana which is one of the countries in southern Africa that aims for malaria elimination by at the latest 2025. Therefore, to reach this milestones, the overall aim of this study was to assess affected citizens' knowledge and preferences regarding malaria vector control methods and, at the same time, to identify and characterize mosquito species as potential carriers of the malaria parasite in Nata village, Botswana.

1.6 Study Objectives

To realize the aim of the study, the following objectives had to be achieved:

- Identify malaria elimination methods employed in Nata village by conducting a descriptive survey;
- Conduct morphological and molecular identification and characterization of mosquito species in Nata village;

- Detect the presence of the *Plasmodium* species harboured by mosquitoes in Nata village using species specific PCR assays.

1.7 Structure of the Study Report

This study report is presented in chapters that comprise the following information:

- **Chapter 1**: General introduction outlining the prevalence of malaria in Botswana, statement of the problem, and study aim and objectives.
- **Chapter 2**: A review of the related literature that was consulted.
- **Chapter 3**: Study methodology and design: Administration of the questionnaire to determine the knowledge and preferences of villagers pertaining to malaria control interventions in Nata village, Botswana.
- **Chapter 4**: Study methods using PCR to detect the *Plasmodium* species harboured by mosquito specimens collected at Nata village. This chapter also focuses on the morphological and molecular identification and characterization of the mosquito species found in the study area.
- **Chapter 5**: General conclusion, summative remarks, and recommendations for future research.

1.8 Conclusion

Chapter 1 provided a general introduction to this study report by highlighting the prevalence of malaria in sub-Saharan Africa with particular reference to Botswana. The threat that malaria-carrying mosquito species pose to human health was described, and it was argued that this study would be vital in providing the authentic views of villagers in Botswana who are at risk of contracting malaria so that more effective prevention strategies could be devised to curb and eventually eradicate the malaria threat in Botswana by 2025.

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CHAPTER 2

LITERATURE REVIEW

2.1 Background of Malaria as a Parasitic Disease

Malaria is a major parasitic disease in developing countries, particularly sub-Saharan Africa. More than half of the world population lives in malaria endemic areas. Globally, 214 million clinical malaria cases and 438 000 deaths have been reported in 2015 (Namuyiga *et al*., 2017; Matangila *et al*., 2017). The authors further highlight that most deaths (90%) occurred on the African continent. Presently, the development and implementation of interventions for the prevention and treatment of malaria focus on the most vulnerable population groups, which are pregnant women and children younger than 5 years (Matangila *et al*., 2017).

It is in this light that WHO adopted four principles that are referred to as the ABCD of malaria. These principles are outlined to protect travellers against contracting malaria:

- A: Be **Aware** of the risk and the symptoms and understand that malaria is a serious infection.
- B: Avoid mosquito **Bites.**
- C: Take **Chemoprophylaxis** when appropriate.
- D: Seek immediate **Diagnosis** and treatment if fever develops during or after travel.

In 2012, 109 countries recorded success with regards to malaria elimination. Of these 109 countries, 34 have on-going malaria elimination processes (Koita *et al*., 2013). In September 2015, Newby *et al*. (2016) state that there were 35 countries that met the malaria-eliminating criteria with national or regional elimination targets ranging from

2013 to 2035. However, a major challenge for the 35 malaria-eliminating countries is the threat of malaria importation. For example, four countries in southern Africa, namely Botswana, Namibia, South Africa and Swaziland, are seeking to eliminate local transmission within the next five years, but many of their neighbours have much higher malaria burdens. The common goal that these countries aim to achieve is malaria elimination by 2020. Moreover, it has been observed that from 2000 to 2013, these 35 countries reduced their malaria burden by a remarkable 90%, meaning that 1.6 million reported cases were reduced to 160 000 cases and, as a consequence, malaria deaths decreased by 87% (Gates and Chambers, 2015). In 2013, eight countries reported fewer than 800 cases of malaria deaths, namely Botswana, China, the Dominican Republic, Iran, Mexico, Panama, South Korea, and Swaziland. These countries have set elimination targets ranging from 2017 to 2025 (WHO, 2015).

Mobile and migrant populations travelling across international borders often serve as primary sources of imported malaria cases, and this phenomenon is likely to drive secondary transmission (Sturrock *et al*., 2015). Report by Tatem and Smith (2010) state that the four main malaria-eliminating countries in the southern African region all reported increases in malaria cases and malaria associated deaths between 2012 and 2013, and they argue that these incidences could possibly be attributed to imported malaria.

The importation of malaria from high-endemic neighbours is a common challenge across most of the 35 malaria-eliminating countries because many of those that have reduced local transmission from the year 2000 have also recorded an increase in imported cases. Indoor house-spraying with DDT, carbamates, or pyrethroids used alone or with ACTs has substantially improved malaria control in some southern African countries that have unstable malaria patterns (Greenwood *et al*., 2005). The same author also emphasized that the formation of the Global Fund against AIDS, Tuberculosis and Malaria was an important first step in the fight against malaria, and this organisation committed nearly US$1 billion to malaria control over a period of two years. Strategies to eliminate malaria target areas where the interruption of transmission is most feasible. The initial target areas included the southernmost margins of endemic malaria in Africa is countries such as Botswana, Namibia, South Africa and Swaziland. The natural limits of *Anopheles* mosquitoes are found in the region of the Pacific islands. Furthermore, Feachem and Sabot (2008) argue that malaria has natural margins that are determined largely by latitude, altitude, and climate and, in some cases, successful human intervention. As geographic latitude and altitude increase, transmission becomes less efficient and malaria less prevalent.

2.2 Malaria Epidemiology

Malaria, especially the infection caused by *P. falciparum,* remains one of the most important causes of morbidity and mortality among impoverished communities across endemic regions of sub-Saharan Africa (Mabunda *et al*., 2008).

In many parts of the world, adequate treatment of malaria is becoming increasingly difficult due to an increase in drug resistance, which has rendered cheap drugs such as chloroquine and sulphadoxine/ pyrimethamine ineffective. Hence prevention of malaria by vaccines is perceived as a tool that will complement currently available strategies for malaria control. In light of more malaria vaccine candidates becoming available, a suitable chosen area with detailed epidemiological information is required for testing and evaluating different trial end points (Mmbando *et al.*, 2009). The site needs to be prepared and should focus on characterizing the population (e.g., its size and structure), accessibility of the site, availability of health services, and the malaria burden in the area. Site preparation involving local communities also requires consideration of ethical issues which, if not adhered to, could compromise the implementation and effectiveness of the trials (Mmbando *et al*., 2009).

As stated by Shaukat *et al*. (2010), vector control success against malaria is based on previous experiences in temperate climate countries where larval control and insecticide spraying of dwellings with dichlorodiphenyltrichloroethane (DDT) resulted in the elimination of malaria from large areas of the globe. Entomological inoculation rate values are used to quantify the impact of insecticide-treated nets (ITNs), indoor residual spraying (IRS), and source reduction (SR) on malaria transmission. ITNs, IRS and SR are the major vector control tools (Shaukat *et al.*, 2010). Certain tools are more appropriate than others depending on the behaviour of the prevalent mosquitoes and environmental factors. All intervention methods require careful planning, trained staff, rigorous supervision and evaluation, free or low-cost access, and sustainability.

2.3 The Transmission and Prevention of Malaria

Malaria is transmitted by the blood feeding of infectious female *Anopheles* mosquitoes, therefore understanding mosquito ecology and population dynamics can inform how best to defeat malaria (Eckhoff, 2011; WHO, 2017). All the important malaria vector species bite (or sting) between dusk and dawn, and the intensity of transmission depends on factors related to the parasite, the vector, the human host, and the environment (WHO, 2017). Transmission is more intense in places where the mosquitoes' lifespan is longer and where there is preference for humans over animals. Transmission also depends on climatic conditions that may affect the number and survival of mosquitoes, such as rainfall patterns, temperature, and humidity. In many places, for example in Botswana, transmission is seasonal with the peak during and just after the rainy season (Eckhoff, 2011).

2.4 Symptoms of Malaria

Malaria can be categorized as uncomplicated or severe (complicated) and, in general, it is a curable disease if diagnosed and treated promptly and correctly (CDC, 2015). Moreover, CDC states that, in most cases, the incubation period varies from 7 to 30 days with shorter periods observed most frequently with *P. falciparum* and the longer ones with *P. malariae.* The symptoms of an uncomplicated malaria infection include fever chills, headache, fatigue, nausea, vomiting, abdominal pains and diarrhoea (WHO, 2017; CDC, 2017).

Complicated malaria symptoms will include acute renal failure, pulmonary oedema, generalized convulsions, and circulatory collapse, followed by coma and death (WHO, 2017; CDC, 2015). WHO elaborates that the initial symptoms of malaria are non-specific and cannot be distinguished from those of other common febrile illnesses such as acute respiratory infections, hence it is important that the possibility of falciparum-malaria is considered in all cases of unexplained fever starting at any time between seven days after the first possible exposure to malaria.

2.5 Diagnosis and Treatment of Patients Afflicted with Malaria

According to (WHO, 2017), diagnosis and treatment, coupled with an effective and safe antimalarial drug, are necessary to prevent life-threatening complications. It is in this regard that WHO recommends that rapid and accurate diagnosis are made, either by microscopy or the rapid malaria test within 24 hours of the onset of a fever in all patients with suspected malaria before treatment is administered.

Rapid and accurate diagnosis of malaria is integral to the appropriate treatment of affected individuals and in preventing further spread of infection among members of communities (CDC, 2015). During microscopic diagnosis, the malaria parasite can be identified by examining a drop of the patients' blood under a microscope. The blood is spread out as a blood smear on a microscope slide (CDC, 2015; WHO, 2017). A rapid diagnostic test (RDT) is an alternative way to quickly establish the diagnosis of malaria infection as this test detects specific malaria antigens in a person's blood (CDC, 2014).

The RDT technique involves applying the patient's blood sample to the sample pad on the test card along with certain reagents which, after 15 minutes, will indicate whether the patient is infected with *P. falciparum* or any of the other three malaria species.

Treatment of malaria should not be initiated until diagnosis has been established by laboratory investigations. Once the diagnosis has been made, appropriate antimalarial treatment must be initiated immediately (CDC, 2013). The treatment depends on the *Plasmodium* species involved, the clinical status of the patient, and the drug susceptibility of the infecting parasites (CDC, 2013). Quinine and its dextroisoner quinidine have been the drugs of last resort for treatment of malaria, while chloroquine has also been used (Bloland, 2001). Antibiotics such as tetracycline are also very potent antimalarial drugs used for both treatment and prophylaxis. Currently, the administration of chloroquine alone as a prophylactic to pregnant females is not recommended in areas where *P. falciparum* resistance to chloroquine is high (Ministry of Health-Botswana, 2015).

A limited number of drugs can be used to treat or prevent malaria, but the most widely used are quinine and its derivatives (Bloland, 2001). Malaria can be prevented through chemoprophylaxis which suppresses the blood stage of malaria infections, therefore preventing the disease (WHO, 2017). However, for pregnant women living in moderate-to-high transmission areas, intermittent preventive treatment with sulfadoxine-pyrimethamine at each scheduled antenatal visit after the first trimester is recommended.

Similarly, for infants living in high-transmission areas of Africa, three doses of intermittent preventive treatment with sulfadoxine-pyrimethamine are recommended. These should be delivered in conjunction with routine vaccinations (WHO, 2017).

2.6 Taxonomy of the Mosquito

Mosquitoes are members of the phylum Arthropoda and belong to the class insect, which is the largest class in the animal group (Triplehorn and Johnson, 2005). Adult mosquitoes can be recognized and characterized by their wing venation, the scales along their wings, as well as their long proboscis. There are some 110 genera and subgenera with 3 500 species of mosquitoes identified and classified under the family Culicidae. This is a large and important group of flying insects (i.e., flies) and their larval stage develops in water. Mosquitoes are two-winged flies that belong to the order Diptera (true flies). The family Culicidae is divided into three subfamilies, namely Anophelinae, Culicinae and Toxorhynchitinae, and they comprise 37 genera. The Anophelinae and the Culicinae are blood feeders but the third subfamily, the Toxorhynchitinae, does not feed on blood (Lehane, 1994).

The genus *Anopheles* contains over 500 species, a few of which are considered important species for malaria transmission. Hence it is important to identify species morphology in order to target scarce resources for controlling malaria vectors (Erlank *et al*., 2018).

The hierarchical structure of mosquito classification is as follow: (Triplehorn and Johnson, 2005):

Kingdom: Animalia

Phylum: Arthropoda

Class: Insecta

Order: Diptera

Family: Culicidae

Subfamilies: Anophelinae, Culicinae, Toxorhynchitinae

Genus: *Anopheles*

2.7 Characteristics of some known mosquito species

Anopheles arabiensis is thus able to utilize a variety of habitats including slow flowing, partially shaded streams and a variety of large and small natural and man-made habitats such as waste, old tyres or any waste that could pose as a favourable place to lay its eggs (Drake and Beier, 2014; Sinka *et al*., 2010).

Studies by Chirebvu and Chimbari (2014) also state that the physical-chemical characteristics of breeding habitats have significant influence on the abundance of *A. arabiensis* mosquito larvae. Sinka *et al*. (2014) conducted a study in Africa, Europe and the Middle East and found that *A. arabiensis* larvae were present in all the areas in small, natural water collections (small streams, seepage spring), small man-made water collections (borrow pits, irrigation ditches) and even in artificial sites (empty cans).

The *A. arabiensis* species is zoophilic, especially when there are animal hosts to feed on. Usually, the females prefer to feed on livestock. The *A. arabiensis* species is thus more likely to prefer outside environments for feeding (exophagic) and these mosquitoes then rest for digestion of blood meals (exophilic) with blood-feeding usually occurring during the night with peak evening biting times beginning in the early evening (19:00) or early morning (at around 03h00) (Tandina *et al*., 2018; Gimba and Idris, 2014; Sinka *et al*., 2010). This group of mosquitoes has as abdomen without laterally projecting tufts of scales, their hind tarsi are pale, and they have palps with pale bands usually with some speckling while their veins have pale spots and their legs are speckled (Gimba and Idris, 2014).

Culicine mosquitoes such as the *Aedes* and *Culex* species are common worldwide and abundantly present in areas where malaria is endemic. Just like the anophelines, they feed on humans and are vectors for human pathogens, transmitting filarial worms and arboviruses such as Yellow Fever, Dengue and the West Nile Virus (Molina-Cruz *et al*., 2013; Knöckel *et al*., 2013). *Culex* is the largest and the most important genus of mosquitoes.

As stated by Ibrahim *et al*. (2018), the *Culex* species is considered as one of the most dangerous insect vectors as it affects humans and animals worldwide through the transmission of highly epidemic and fatal diseases. Although culicines do not transmit human malaria, studies have revealed that they transmit avian malaria (Molina-Cruz *et al*., 2013). The *Culex* species, such as *C. quinquefasciatus* is markedly domestic and these mosquitoes bite people and animals throughout the night.

They roam both indoors and outdoors, the same as *Aedes aegypti* mosquitoes, and bite mainly in the morning and evening (Rozendaal, 1997). The *Culex* species is also regarded as a nuisance as it mainly breeds in organically polluted water such as drains, soakage pits and open sewage systems (Emidi *et al*., 2017). The latter authors conducted their study in Tanzania and revealed that pit latrines were the main sources of *C. quinquefasciatus* as breeding sites. It was also shown by studies in Nigeria that other sites that contributed to increased numbers of species were container-type breeding sites and swimming pools.

A variation in densities of *C. quinquefasciatus* occurred across the seasons of study and its prevalence was associated with the wetness status of pit latrines due to rainfall patterns. During the rainy season, pit latrines tend to overflow due to rain water percolation, which makes them potential breeding sites for *Culex* mosquitoes. The variation in the densities of *C. quinquefasciatus* emerging from pit latrines across the seasons was very high during cool and dry seasons as well as during the long rainy season (Emidi *et al*., 2017a)

Another study by Emidi *et al*., (2017b) on the effects of physicochemical parameters on *Anopheles* and *Culex* mosquito larvae abundance in different breeding sites in a rural setting of Muheza in Tanzania confirmed that this species breeds in various habitats. The research further states that *Anopheles gambiae* breed in small, open, sunlit, fresh water bodies whereas *Anopheles funestus* normally breeds in water bodies with emergent vegetation such as swamps and rice fields. Lastly, *C. quinquefasciatus* breeds in polluted water habitats such as pit latrines soak pits, cesspits and open sewage systems.

Nonetheless, research by Jansen *et al*. (2013) speaks of the advantages of morphological identification techniques over molecular techniques. They state that it may sometimes be difficult to use molecular tools for mosquito identification because of lack of resources and technical limitations, and because costs are high especially when the identification of thousands of mosquito specimens is required. The latter study further emphasizes that morphological identification, even though some studies regard it as slow and time consuming, remains the primary method that can be employed, particularly in field based studies. They state that it as an important tool that field researchers need to be aware of for accuracy of morphological identifications (Jansen *et al*., 2013).

2.8 Morphology of the Adult Mosquito

The body of a mosquito is differentiated into a head, thorax and abdomen with a short and mobile neck joining the head with the thorax (Figure 4.1).

Adult mosquitoes are small (usually about 5 mm long), rather delicate insects with slender bodies, long legs and elongated, forwardly projecting mouth parts (Lehane, 1991). The head of a mosquito is small and spherical in shape and has two large compound eyes and a pair of long, many segmented antennae. Mosquitoes also have a pair of veined wings and a straw-like proboscis and can only consume liquids. The mouth parts of male mosquitoes are adapted to sucking as they feed on the nectar of flowers, while those of females are designed for piercing and sucking as they have to pierce the skin of warm-blooded vertebrate hosts and suck their blood. The body of a mosquito is covered with tiny scales and it appears pale brown or reddish brown.

They have a pair of long and narrow wings with scales along the veins and margins (McGavin, 2001). When resting, mosquitoes hold their single pair of wings over the abdomen like a pair of closed scissors. In most anophelines, the wings have a dappled appearance because of alternating blocks of dark and light scales on the wings. In contrast, most culicines have wings that lack distinct markings. Characteristically, all mosquitoes have scales on their wing veins and the trailing edges of the wings. Adult members of the blood sucking subfamilies (Anophelinae and Culicinae) can be distinguished in the field by their resting postures (Lehane, 1991; McGavin, 2001).

2.8.1 Life Cycle of the Mosquito

The greater part of the life cycle of mosquito occurs in water until the adult emerges and needs to survive in the environment.

The life cycle of mosquito is divided into four distinct stages: egg, larva, pupa and adult (Figure 2.1). Each stage in the life cycle goes through visible morphology.

The life cycle duration varies and is dependent on temperature and food resources. In summer this life cycle usually takes 3 to 10 days to complete it (Renchie and Johnsen, 2007). The *Anopheles* female lays her eggs singly in fresh water and the boat-shaped size of the eggs is about 1 mm. About 100-250 eggs, which are not visible with the naked eye, are laid and these will hatch within 2 – 3 days (Renchie and Johnsen, 2007; CDC, 2015).

After this period, the larvae emerge from the eggs and they go through a fourth-instar period as they gradually increase in size. The body of the larva is divided into three parts: head, thorax and abdomen, with spiracles (respiratory organs) present on the abdomen for breathing (CDC, 2015). The fourth-instar larva will moult into a pupa in 5 − 7 days. This is the stage in the mosquito's life cycle when transition occurs from the aquatic to the terrestrial adult stage. Mosquito pupae do not feed and will moult into adult mosquitoes within 1 − 2 days (CDC, 2015). When the adult emerges from the pupa, the wings need to be fully developed before it can fly away. Mating usually starts as soon as both male and female are fully developed. Normally, the adult mosquito lives for about 2 − 3 weeks (CDC, 2015). This life cycle is graphically presented in Figure 2.1 on the next page.

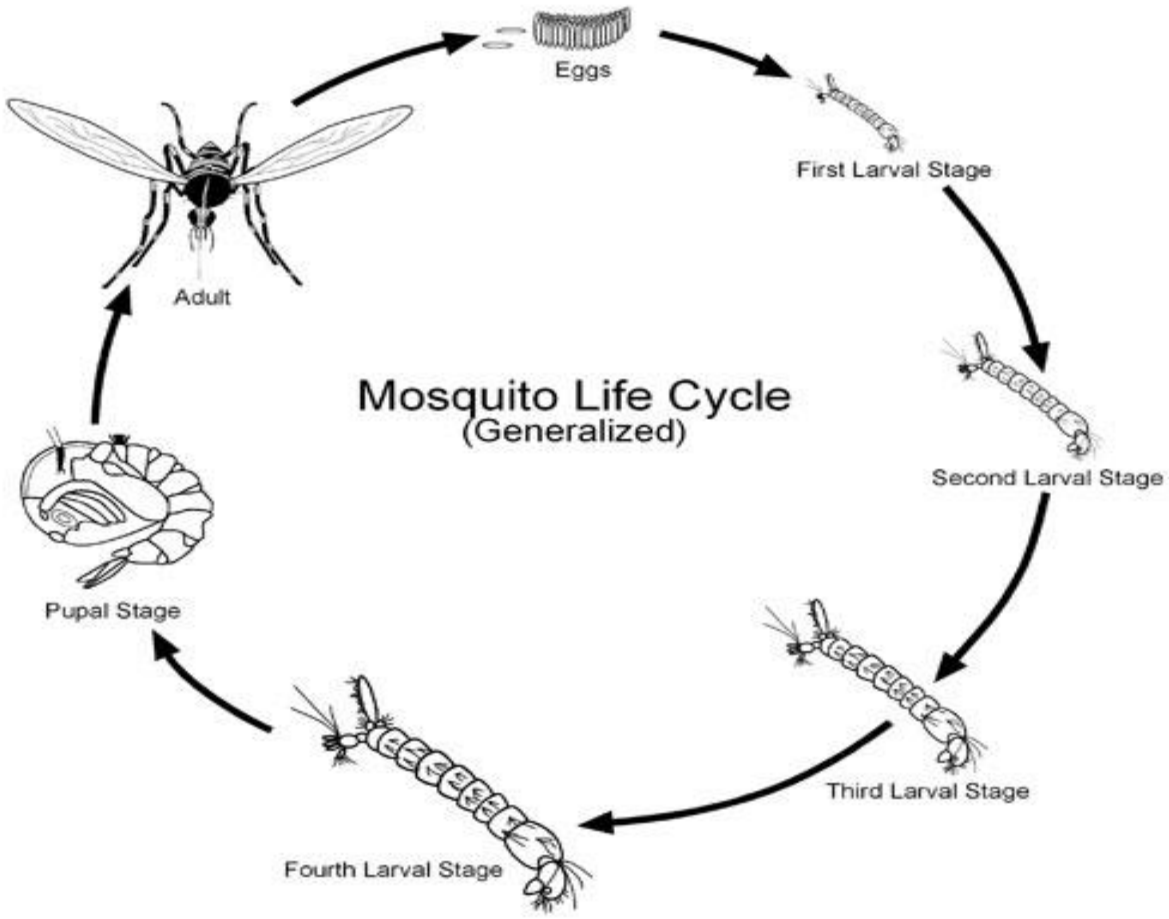

Figure 2.1: Mosquito life cycle; Source: Charlesworth, 2008

2.9 Vector Control of Malaria

WHO recommends long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) as two main methods for malaria vector control (WHO, 2017). Both IRS and the use of LLINs are essential for malaria vector control, but these tools are also challenged by the insecticide resistance that develops in mosquitoes. Malaria vector controls that are utilized by endemic countries through National Malaria Control Programmes (NMCPs) are supported by partners such as the Global Fund (Thiaw, 2018).

It has been argued that effective vector control has the ability to protect all people at risk of malaria (WHO, 2017).

2.9.1 Long-lasting insecticidal nets (LLINs)

An insecticide-treated net (ITN) is a net (usually a bed net) that has been treated with safe residual insecticide and it is designed to block mosquitoes physically. Bed nets form a protective barrier around people sleeping under them and are usually deep treated using synthetic pyrethroids such as deltamethrin or permethrin to enhance their protection ability. The nets are designed to remain effective for at least 3 years even after repeated washing, hence the term 'long-lasting insecticidal nets' (LLINs) (CDC, 2015). The use of LLINs is a key vector control intervention that blocks exposure to potentially infective mosquitoes and prevents contact between a mosquito and a malaria-infected person. Ultimately, the insecticide in these nets kills mosquitoes should contact be made (Levitz *et al.,* 2018). LLINs are responsible for preventing the transmission of malaria and studies have shown that they were able to reduce morbidity by 50% generally and under-5 mortality by 17% in endemic areas (Kanyangarara *et al.,* 2018; Levitz *et al.,* 2018). For a high level of protection against malaria, the physical state of the LLINs is very important, because if they are poorly maintained they can last for only a year, after which their protection ability against malaria is reduced (Quive, 2015). Mass campaigns for the free distribution of these nets have resulted in a rapid increase of LLIN ownership and use. Across sub-Saharan Africa, the use of LLINs has been shown to be associated with an average parasite prevalence reduction of 20% (Ntuku *et al.*, 2017).

Sustained high usage rates of LLIN and other effective interventions are essential for the achievement and maintenance of a reduction in the malaria burden and the ultimate achievement of the target of a zero malaria threat. Campaigns that launched the mass distribution of these nets have thus resulted in rapidly increase in LLIN ownership and use in several countries. Across Africa, different distribution strategies such as fixed or door-to-door delivery have been used with varying effects on LLIN coverage and use (Larson *et al*., 2014). Moreover, community-wide use of LLINs can affect vector density and longevity over large areas. This strategy forces the *Anopheles* mosquito to find alternative non-human hosts and, ultimately, this reduces mosquito survival and malaria transmission (Haji *et al*., 2013).

Pregnant women are particularly vulnerable to infection and this poses serious health outcomes for both the mother and her unborn child. The risk can be mitigated through appropriate use of control measures such as insecticide-treated bed nets (Ernst *et al*., 2017). Since the turn of the century, the number of malaria deaths has been reduced by nearly half, and this progress is attributed to the increased distribution and use of LLINs, which have been shown to be one of the most effective tools to combat the disease as they provide both individual and communal benefits (Ernst *et al*., 2017).

2.9.2 Indoor residual spraying (IRS)

The IRS involves coating the walls and other surfaces of a house with a residual insecticide. The insecticide will be effective in killing mosquitoes and other insects for several months including when they come into contact with these surfaces.

IRS does not directly prevent people from being bitten by mosquitoes, but it kills mosquitoes after they have fed and should they come to rest on the sprayed surface. Indoor spraying is effective for 3 – 6 months, depending on the insecticide formulation used and the type of surface on which it is sprayed (CDC, 2012; WHO, 2017). This prevention method targets adult vectors and it involves spraying the inner walls of houses with WHO-approved insecticides that contain residual properties. Once applied, these insecticides dry up, leaving a small film of crystals on the wall. The vector picks up the insecticide when it rests on the surface either before or after a blood meal and, if susceptible to the insecticide, it dies from the exposure to it. Some of the insecticides used for IRS are also able to repel mosquitoes and this reduces the number of vectors entering sprayed rooms (Williams and Pinto, 2012). Indoor residual spraying (IRS), combined with insecticide treated nets (ITN), has been implemented in several sub-Saharan countries with inconclusive evidence on the effect of this combined intervention strategy (Protopopoff *et al*., 2015). Over the past decades, the beneficial effects of IRS on malaria prevention have been reported in both high and low malaria endemic areas. IRS has contributed to the elimination or dramatic reduction of malaria in various parts of Latin America, Asia and Europe.

However, it is important to note that community knowledge of IRS activities increases the likelihood of this strategy being fully accepted in malaria prevention programmes. Therefore, only if communities are well informed and have 'bought into' such programmes, high IRS coverage will be achieved as envisaged by the Ministry of Health's National Malaria Control Programme (Chirebvu *et al*., 2013).

It is in this context that Indoor residual spraying, together with use of insecticide treated nets, has been scaled up and implemented in 31 countries across Africa. The rationale for combining these two intervention strategies is to increase coverage and accelerate reduction of malaria, as the use of only one intervention strategy has not had the desired effects. Furthermore, the combination of these two methods can compensate for the loss of effectiveness should one intervention become compromised. For example, when insecticide resistance occurs or when IRS is waning off (Protopopoff *et al*., 2015), the other will still function effectively. IRS was proposed as the main strategy by the Global Malaria Eradication Campaign, which resulted in the elimination of malaria from many countries and greatly reduced the malaria burden in others. However, IRS has traditionally been targeted in low and/or seasonal transmission areas, and its recent expansion into high transmission areas has been questioned due to concerns about long- term sustainability (Gimnig *et al*., 2016).

2.9.2.1 The use of dichlorodiphenyltrichloroethane (DDT) in the control of malaria

Mosquito control has been at the centre of past efforts to eradicate malaria, and this occurred mainly through the use of the insecticide DDT for indoor residual house-spraying (Greenwood *et al*., 2005). However, due to its highly toxic effect of the environment, DDT was banned under the 2001 Stockholm Convention on persistent organic pollutants for all uses except disease control. But, in 2006, WHO again endorsed the use of DDT for vector control in malaria endemic areas under strict regulations (Eskenazi *et al*., 2009).

Although approximately 123 million people may be exposed to high levels of insecticides through the use of indoor residual spraying (IRS) for malaria control, few studies have been conducted on indoor insecticide contamination due to IRS and its relationship with human exposure. Nonetheless, indoor residual spraying and the application of insecticides to interior walls, ceilings and eaves as proposed by malaria vector control policies were adopted by 88 countries (Gaspar *et al.*, 2015).

The DDT is currently used for malaria control in at least ten countries, including Botswana, the Democratic Republic of Congo, Gambia, India, Mozambique, Namibia, South Africa, Swaziland, Zambia and Zimbabwe. DDT is used in these countries as an IRS constituent because it has a long residual efficacy (>6 months) and non-contact spatial repellent properties (WHO, 2014; Gaspar *et al.*, 2015). Animal studies have shown that DDT and its breakdown product, dichlorodiphenyldichloroethylene (DDE), are neurodevelopmental toxicants. DDT levels timed to sensitive periods of prenatal and neo-natal nervous system development in mice have been shown to cause behavioural and neurochemical changes in adulthood (Gaspar *et al*., 2015). Recently, it was reported that high concentrations of DDT residues have been detected in human milk and blood from inhabitants in Limpopo Province in South Africa as well as in terrestrial and aquatic bird eggs from the same area (Viljoen *et al*., 2016). These findings are naturally a matter of grave concern, as the continued use of DDT may exacerbate the threat that it poses for humans and the environment.

2.9.2.2 Pyrethroids

Malaria vector control is currently highly dependent on a single class of insecticides, namely pyrethroids. These insecticides are the only class approved for use on insecticide-treated nets and are being increasingly deployed in IRS programmes in Africa. Some insecticides, including these synthetic pyrethroids, also repel mosquitoes from households, leading to reduced human mosquito contact and increased mortality rates of adult mosquitoes (Ranson *et al*., 2011; Skarbinski *et al*., 2012). Synthetic pyrethroids are being used extensively for indoor residual sprays, space sprays and in impregnated bed nets. Some of the recommended pyrethroids include permethrin, deltamethrin and lambdacyhalothrin (Table 2.1). These pyrethroids are highly effective only if optimally applied, but resistance to these chemicals reduces their impact and personal protection measures are required with the use of insecticide-treated nets (ITNs), especially when synthetic pyrethroids were used (Tiwari *et al.,* 2010). The use of pyrethroids for IRS constitutes one of the major interventions for the reduction and interruption of malaria transmission by vector control in all epidemiological settings (Feo *et al*., 2012). Moreover, the use of pyrethroid insecticides in malaria vector control has increased dramatically in the past decade, particularly as they have been applied in insecticide-treated net distribution programmes and indoor residual spraying campaigns (Ranson *et al*., 2011). However, recently mosquito resistance to pyrethroids has emerged in many countries. In some areas, resistance to all four classes of insecticides used for public health has been detected. Fortunately, this resistance has only rarely been associated with decreased efficacy of LLINs, which continue to provide a substantial level of protection in most settings.

Rotational use of different classes of insecticides for IRS is recommended as one approach to manage insecticide resistance (WHO, 2017).

Table 2.1: WHO recommended insecticides for indoor residual spraying (IRS) against malaria vectors

Insecticide compounds & formulations	Class group	Dosage (g a. i./m^2)	Mode of action	Duration of effective action (months)
DDT WP	OC	1-2	Contact	> 6
Malathion WP	OP	2	Contact	2-3
Lambdacyhalothrin WP, CS	PY	0.02- 0.03	Contact	3-6
Fenitrothion WP	OP	2	Contact & airborne	3-6
Pirimiphos-methyl WP & EC	OP	1-2	Contact & airborne	2-3
Bendiocarp WP	C	0.1-0.4	Contact & airborne	2-6
Propoxur WP	C	1-2	Contact & airborne	3-6
Alpha-cypermethrin WP, SC	PY	0.02- 0.03	Contact	4-6
Bifenthrin WP	PY	0.025- 0.050	Contact	3-6

Cyfluthrin WP	PY	0.02- 0.05	Contact	3-6
Deltamethrin WP, WG	PY	0.020- 0.025	Contact	3-6
Etofenprox WP	PY	0.1-0.3	Contact	3-6

CS: capsule suspension; EC = emulsifiable concentrate; SC = suspension concentrate; WG = water dispersible granule; WP = wettable powder; OC = organochlorine; OP = organophosphate; C = carbamates; PY = pyrethroid

Source: WHO, 2013

2.9.2.3 Lambdacyhalothrin as an alternative pyrethroid

Lambdacyhalothrin (Icon) is a pyrethroid that consists of a racemic mixture of the two most active of the four isomers of cyhalothrin. It is stable on a variety of inert surfaces and can be used for vector control as it is active against insects that are resistant to organophosphates and carbamates. This pyrethroid is one of the main insecticides used in malaria vector control in indoor residual spaying (IRS) by most countries in southern Africa. It works by disrupting the normal function of the nervous system of a mosquito (NPIC, 2001; Moretto, 1991). Icon 10 WP, a wettable powder formulation containing 10% of the pyrethroid insecticide lambdacyhalothrin, is widely used by countries in the southern African region (such as Botswana) in conjunction with DDT (Chester *et al.,* 1992). Synthetic pyrethroids are safe alternatives to DDT, especially when sprayed onto modern housing surfaces and when used in low and seasonal transmission areas. In Africa, sustained IRS has historically been used mainly in the southern part of the continent where it has been successful in controlling malaria and reducing transmission. With current efforts to up-scale malaria control in Africa, IRS has been introduced in a number of countries with initially high levels of transmission (Mashauri *et al.,* 2013).

Furthermore, Mashauri *et al.* (2013) argued that IRS with lambdacyhalothrin is still one of the most effective intervention measures to stem malaria in epidemic prone rural areas. Malaria vectors that are controlled with the use of IRS containing lambdacyhalothrin is the first large-scale intervention and initial malaria parasitological results are encouraging as the results are consistent with those of other IRS interventions in sub-Saharan countries.

2.9.3 The presence of insecticides in food products

There is an assumption that pyrethroid pesticides are converted to non-toxic metabolites by hydrolysis in mammals. However, some recent studies have shown that their bioaccumulation in human breast milk collected in areas where pyrethroids have been widely used for agriculture or malaria control (Corcellas *et al*., 2012). The presence of pyrethroid insecticides in human breast milk and in thatch wall material of dwellings in southern African subtropical areas (Manhiça, Mozambique) has been investigated to assess the potential pyrethroid route to hazardous human exposure, particularly because breast milk is the primary source of infant nutrition and exposure to DDT through breast feeding is a matter of grave concern (Feo *et al*., 2011). Study by Corcellas *et al*. (2012) argue that, because the use of pyrethroids has increased widely as indoor household insecticides such as insect-control products, pet shampoos and lice treatments as well as outdoor as agricultural pesticides and for pest control, their use and effect should be rigorously monitored.

In some individuals, pyrethroid levels were higher than DDT levels, which suggest domestic and home garden use of the former, while the presence of DDT was attributed to activities for control of malaria vectors (Feo *et al*., 2011). Restrictions on or the banning of many of these compounds for agricultural use in most parts of the world has led to gradual reductions in the levels of organochlorine compounds in breast milk (Bouwman *et al*., 2006). Human exposure to pyrethroids, including lambdacyhalothrin, can occur via various routes, including dermal uptake, inhalation (dust and gas phase) and ingestion of food containing residues of this group of insecticides.

The use of these compounds in agriculture and for IRS may therefore explain their occurrence in the milk samples collected in some studies (Feo *et al*., 2011). It is highlighted by Bouwman *et al*. (2006) that in many rural areas of Africa, subsistence foodstuffs are produced close to homesteads and supplemented with food from markets and shops. Findings by previous studies have indicated that traces of DDT that had been used for indoor residual spraying manifested in food (including chicken meat and fat), soil, water and air, and they argue that these were likely contaminated by this insecticide (Bouwman *et al*., 2006). Such findings suggested that the uncontrolled and irresponsible use of this pesticide is cause for severe concern and that strong measures should be taken to regulate and control its usage for malaria control.

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CHAPTER 3

KNOWLEDGE AND PREFERENCES OF MALARIA CONTROL INTERVENTIONS AMONG VILLAGERS IN NATA VILLAGE, BOTSWANA

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3.1 Abstract

Long-lasting insecticide nets (LLINs) and indoor residual spraying (IRS) are the main tools for malaria vector control in most countries in sub-Saharan Africa, including Botswana. LLINs might be preferred over IRS and repellents, but this does not suggest that they should replace IRS and repellents in all cases or be used alone, because the resistance of vectors to pyrethroid insecticides used for LLINs is high and a combination of methods is often required to address this problem.

Dichlorodiphenyltrichloroethane (DDT) and pyrethroids are the main insecticides used for malaria vector control in most southern African countries. The aim of this phase of the study was to document information on Nata villagers' knowledge of malaria control interventions and to assess their perceptions and preferences regarding the use of methods to curb malaria in the study area. A descriptive cross-sectional study was conducted utilizing 109 residents of Nata village in Botswana. An interviewer-administered questionnaire was used to collect the required data on the methods the villagers preferred to use for malaria control interventions. About 83% of the participants had knowledge of the use of LLINs to control malaria whereas, respectively, 21% and 9% had knowledge of IRS and other repellents. Only 8% of the participants preferred both IRS and repellents and 12% preferred both the LLIN and IRS methods, whereas only 1% demonstrated concurrent preference for LLINs, IRS and repellents for maximum protection against mosquitoes and malaria.

Despite a fair knowledge of malaria control interventions by the majority of the participants, the findings suggest that there is a need for continuous education on the importance of using both LLINs and IRS for the prevention and elimination of malaria in the study area.

Keywords: Malaria, LLINs, IRS, DDT, pyrethroids, preference, knowledge

3.2 Introduction

Malaria has been a persistent and major public health burden in many regions globally and is the leading cause of morbidity and mortality across the globe. Furthermore, it remains an impediment to socio-economic growth and welfare, particularly in rural areas (Sumari *et al*., 2016). The SADC (2017) malaria report states that there were 60 414 malaria related deaths in 2016 in southern Africa. The success of intervention programs such as LLINs depends in the cooperation of the community and their understanding that different intervention control methods should be used to curb malaria. Methods to eradicate mosquito breeding sites such as stagnant water and dense vegetation (i.e., environmental management processes) have long term implications and have been advocated in many countries (Dlamini *et al*., 2017). However, any intervention for reducing the burden of malaria and other diseases depends on the level of consumers' knowledge about malaria and methods to control it and this can only be achieved through education and community mobilization on malaria prevention and control measures (van ben Berg *et al*., 2018).

Education about and knowledge of malaria are critical in sensitizing the perceptions and modifying the practices of communities regarding malaria control. The success of malaria control efforts is thus dependent on citizens' level of understanding, their attitudes, and socio-cultural aspects of malaria prevention within affected communities (Sumari *et al*., 2016).

Apart from the availability and affordability of treatment options, there are also various other factors within communities that will impact their efforts to effectively combat malaria. Usually the decision on whether to seek treatment is influenced by how well explanations have impacted understanding of the disease (Dlamini *et al*., 2017). Therefore, the fact that the use of LLINs and other interventions such as indoor residual spraying (IRS) should coverage in malaria control is very important in planning and assessing intervention campaigns and national control strategies. Even though the use of LLINs is an intervention that target individuals, enough of these nets should be supplied to the community in order to achieve the desired outcome. For example, assessing the impact of an abundant LLIN supply to the community has shown a decreased risk of malaria (Levitz *et al*., 2018). Sumari *et al*. (2016) also found that a decline in malaria risk was achieved by the implementation and roll-out of various malaria interventions in affected countries. Reports on the knowledge, attitude and practices relating to malaria and its control interventions are available from different malaria endemic African countries. Most of these reports conclude that misconceptions concerning malaria still exist and practices to control malaria are still unsatisfactory (Sumari *et al*., 2016).

Botswana, South Africa, Namibia and Swaziland are on the verge of eliminating malaria by implementing malaria control activities that include routine vector control and knowledge of transmission dynamics (Tawe *et al*., 2017). However, the dissemination of information regarding mosquito control techniques to curb malaria is complex and it involves several technical, operational, economic and social factors. When introducing mosquito control techniques, it is vital that they should be appropriate for recommended settings and the environment where they will be used. It is therefore advisable to seek the opinions of the community that will be using the recommended methods in order to improve their distribution and effective use (Smith *et al*., 2011). The methods that are preferred by communities are likely to vary; some may prefer bed nets (treated or not) while others may prefer treated nets over untreated nets. The community's preferences for malaria technologies also have implications for the development of new technologies. Therefore, the implication is that there will be different categories of users based on malaria control technique preference (Smith *et al*., 2011).

3.3 Materials and Methods

3.3.1 Study area

This study was conducted in Nata village (20.2105° S, 26.1773° E) in the Tutume District in northern Botswana (Figure 3.1). The village is situated in a popular tourism area where most of the residents are subsistence farmers. The village residents and the farmers are involved in tourist attraction activities such as providing visitor accommodation, site seeing (game drives) and the creation and selling of traditional artefacts.

Nata village has seven wards where malaria is transmitted year-round with a seasonal peak during the rainy period from October to March (BOPA, 2014).

Figure 3.1: Map of the Republic of Botswana showing the study area (circled)

Source: www.lahistoriaconmapas.com/atlas/country-map03/botswana-nata-map.htm

3.3.2 Study design and data collection methodology

This part of the study comprised a descriptive cross-sectional survey to assess the knowledge and preferred methods of malaria control among the citizens of Nata village.

An interviewer-administered questionnaire was used to gather information on selected villagers' malaria control methods, knowledge and preferences.

The first part of the questionnaire elicited demographic characteristics and the second part required responses to four questions on the respondents' knowledge, preference and perceptions pertaining to malaria control methods.

The questionnaire was administered to 109 randomly selected volunteer participants during two malaria seasons (November 2017 and February 2018) for 5 days per season. Descriptive statistical tests were used to analyse the data using Microsoft Excel version 2013.

3.3.3 Ethical considerations

The study proposal was approved by the Health Research and Development Division under the Ministry of Health and Wellness of Botswana (Ref no. HPDME 13/18/1) and the Faculty Research Committee of the Central University of Technology, Free State (South Africa). The questionnaire was developed in the Setswana and English languages and piloted before being administered. Each participant signed a consent form and had the choice to answer either in Setswana or English.

3.4 Results

3.4.1 Demographic characteristics of the participants

Of the 109 community members who participated in the study, 71 (65%) were females and 38 (35%) were males (Table 3.1).

The ages of the participants ranged from 17 to 82 years with mean and standard deviations of 34 and 13 respectively. An inclusion criterion was that participants had to be 17 years or older to take part in the study.

Table 3.1: Demographic characteristics of the study participants (N=109)

Gender	Frequency	Percentage (%)
Female	71	65
Male	38	35

3.4.2 Knowledge of use of available and preferences of malaria control initiatives among villagers in Nata, Botswana

All the participants (100%) had knowledge of malaria control initiatives as implemented by the government in their village, including those methods that are for sale at retail shops for their protection. For example, they were aware of mosquito repellents.

Generally, the participants mentioned long lasting insecticidal nets (LLINs), indoor residual spraying (IRS) and repellents as the malaria control methods they were familiar with in the following order: 91 (83%) mentioned long lasting insecticidal nets, 23 (21%) mentioned only indoor residual spraying, while 10 (9%) referred only to repellents as a familiar malaria intervention strategy (Table 3.2). The participants noted their preference for one or more methods of malaria control: 9 (8%) preferred only repellents, 9 (8%) preferred only IRS; while a majority of 77 (71%) preferred only LLINs. Combined methods were preferred by 13 (12%) respondents who used both IRS and LLINs.

Only 1 (1%) participant preferred all three the malaria control methods namely IRS, repellents and LLINs (Table 3.3).

Table 3.2: Participants' knowledge on use of available malaria control methods (N=109)

Method	Frequency	Percentage (%)	Cumulative Frequency	Cumulative Percentage
LLINs	18	17	18	17
	91	83	109	100
IRS	86	79	86	79
	23	21	109	100
Repellents	99	91	99	91
	10	9	109	100

Table 3.3: Participants' preferences of malaria control methods

Method 1	Method 2	Method 3	Frequency	Percentage (%)	Cumulative Frequency	Cumulative Percentage
		Repellents	9	8	9	8
	IRS		9	8	18	16
LLINs			77	71	95	87
LLINs	IRS		13	12	108	99
LLINs	IRS	Repellents	1	1	109	100

3.4.3 Participants' recommendations regarding malaria control methods to be used (LLINs, IRS, repellents)

Of the 109 participants, more than half (74/68%) did not express any views regarding recommendations for the use of malaria control methods, whereas 30 (27%) supported an increased supply of LLINs. Conversely, only 2 (2%) suggested a free supply of repellents by the government instead of them having to buy them. Similarly, 2 (2%) suggested that IRS, LLINs and repellents be used concurrently for protection against malaria vectors. Only 1 (1%) suggested the increase of indoor residual spraying coverage for effective malaria control (Table 3.4).

Table 3.4: Participants' recommendations regarding the use of LLINs, IRS and repellents

Views	Frequency	Percentage	Cumulative Frequency	Cumulative Percentage
All to be used together	2	2	2	2
IRS coverage to be increased	1	1	3	3
Supply of Repellents	2	2	5	5
None	74	68	79	73
Supply of LLINs to be increased	30	27	109	100

3.4.4 The role of insecticides in the contamination of the environment and food

The insecticides used in Botswana to control malaria vectors are predominantly DDT and pyrethroids. DDT is usually used in traditional structures whereas pyrethroids are used for modern structures. During indoor residual spraying, all food products are supposed to be removed from the house to prevent contact with the insecticides. In this study, the participants were asked if they had any idea whether the insecticides that are used for indoor residual spraying, in LLINs and as repellents contaminate food and food products.

Only 12% (Figure 3.2) believed that insecticides contaminated food, whilst 88% had no knowledge of possible food contamination by insecticides.

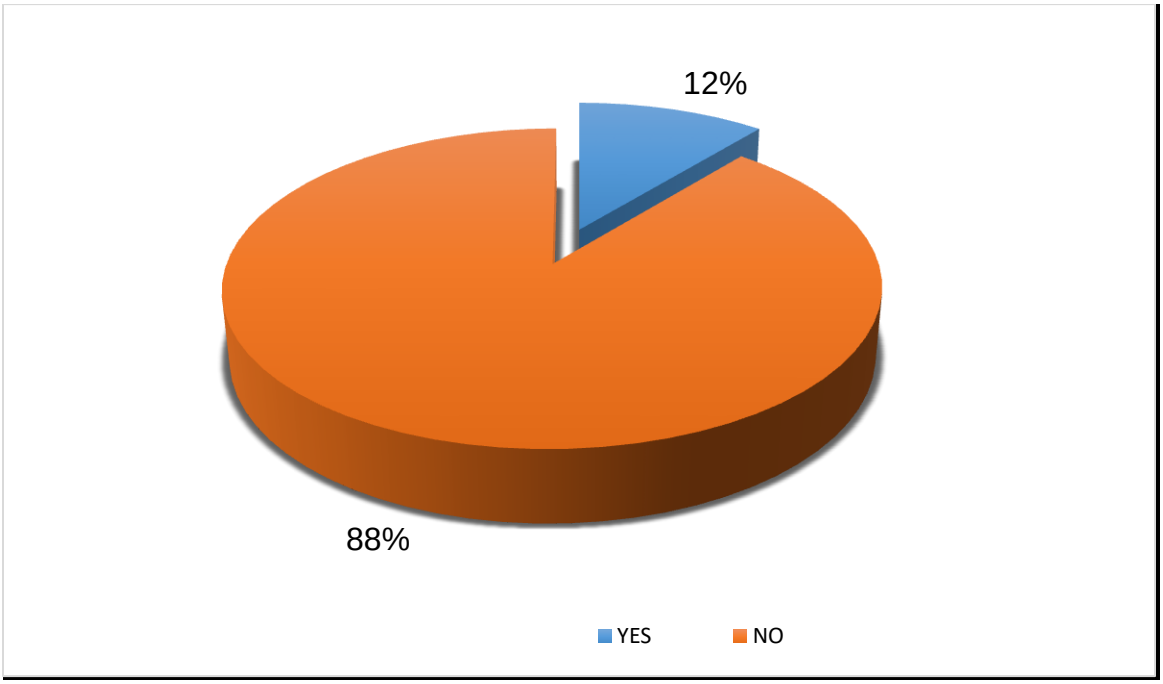

Figure 3.2: Knowledge of insecticide contamination of food products

3.5 Discussion

The data showed that all the participants (100%) had knowledge of methods used for malaria control as implemented by the Botswana government under the malaria control and prevention programme. A study by Hlongwana *et al*. (2009) found that, apart from the use of LLINs for protection against malaria, their participants preferred repellents such as mosquito coils and other traditional methods such as the burning of cow dung or leaves (3.4%), while others preferred closing of windows and doors (6.3%).

In the current study, some of the participants mentioned the use of traditional methods such as burning cow dung or tree leaves to complement indoor residual spraying (IRS), long lasting insecticidal nets (LLINs) and repellents. These additional methods require further scientific investigation to determine their effectiveness because the success of malaria control programmes relies heavily on community perceptions and practices related to the transmission, treatment and control of malaria. More especially, if inappropriate beliefs and behaviours impact the community's efforts to control malaria, they will interfere with the effectiveness of confirmed control measures (Nwakwasi *et al*., 2017). The current study found that the majority of the participants (83%) preferred LLINs, but their effectiveness decreases over time due to wear and tear (Lee *et al*., 2017) and the diminishing effectiveness of the repellent they contain. This finding suggests that, if such nets are not replaced on a continuous basis, they will become ineffective in mosquito control which, in turn, will leave unsuspecting users exposed to infection. This is particularly disconcerting as the use of LLINs was the most preferred method over IRS and repellents. Study by Lee *et al*. (2017) also caution against the exclusive use of these nets, arguing that they cannot completely replace IRS because of their high resistance to pyrethroids. Mixed perceptions about IRS and its acceptability have been noted by Lee *et al*. (2017) related to its perceived benefits in terms of the reduction of mosquitoes numbers, nuisance, biting, and malaria. Conversely, the current study documented a low percentage (21%) preference for IRS alone, which was attributed to health reasons and conditions such as asthma and skin irritation.

In study by Bouwman *et al*. (2012), they argue that many options to improve IRS remain to be explored while maintaining effective transmission prevention and the reduction of human exposure to IRS chemicals. The latter authors also highlight that IRS is effective in preventing transmission, but ineffective in preventing chemical exposure and uptake by residents. Moreover, Manokari and Meenu (2014) state those commercial insect repellents are recommended as the most effective form of bite-preventive treatment, although only 9% of the participants in the current study preferred repellents only. In this context, West *et al*. (2014) reiterate that IRS, when used in combination with LLINs, has the potential to give significant added protection against malarial infection compared to LLINs use alone. A study by Simon *et al*. (2013) that was also conducted in Botswana revealed that the use of both LLINs and IRS had reduced new cases of malaria by approximately 98% and the number of deaths from 12 to 3 in the period preceding their study, therefore they argue that this combined method makes a positive contribution towards eliminating malaria. The finding by the current study that some of the participants preferred the use of one or more malaria control methods to protect themselves against malaria and mosquito bites was therefore encouraging; however, the percentages that preferred more than one method were relatively low, which suggests that much should still be done to inform and sensitize citizens regarding the use of multiple methods to curb the treat of malaria infection. For example, only 12% of the participants preferred the use of LLINs and IRS while only 1% preferred the combined use of LLINs, IRS and repellents.

According to Okumu and Moore (2011), the universal use of LLINs in conjunction with IRS is actively promoted as the main prevention strategy under the WHO endorsed malaria control and elimination plan, but even though a combination of LLINs and IRS is increasingly being practised, there is insufficient evidence as to whether it is indeed better than LLINs or IRS alone (Okumu and Moore, 2011). In this study, 71% of participants preferred the use of LLINs alone.

It was a disconcerting finding that 88% of the participants in the current study did not know whether the insecticides used for malaria control during indoor residual spraying could contaminate food, and that only 12% knew that insecticides posed some threat for the contamination of food. A study by Bouwman *et al*. (2015) found traces of insecticide in chicken eggs which undoubtedly posed a risk to consumers. The latter study also cautions that, in malaria endemic areas where DDT is still used for vector control through indoor spraying, the concentrations of DDT in human blood and breast milk are high, and this negatively impacts human health.

3.6 Conclusion

The villagers' knowledge of malaria control measures and their preference for the use of long lasting insecticide nets were satisfactory. However, their attitudes towards and their practices of the use of malaria control interventions such as LLINs and IRS still need improvement through information dissemination and education strategies. Information sharing strategies and the inclusion of community members in policy making for specific approaches to malaria control and prevention should therefore be addressed.

Education should occur through the use of different media such as the radio, television and the printed media so that all affected community members, including school children, could be reached. Only when collaboration and a holistic approach are used, will strategies for malaria control and elimination be broadened and rolled out. Moreover, even though the school curriculum may include topics on diseases that will benefit young people, it will be wise to educate people above 50 years of age because they have the responsibility to adapt malaria control interventions for the benefit of their families and the community as a whole (Dlamini *et al*., 2017).

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CHAPTER 4

MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF MOSQUITOES IN NATA VILLAGE, BOTSWANA

4.1 Abstract

Mosquitoes are known as vectors of *Plasmodium* parasite which causes a disease called Malaria, which is a major parasitic disease in developing countries and particularly sub-Saharan Africa. More than half of the world population lives in malaria endemic areas. Vector control has been one of the effective strategies for prevention or reduction of malaria infection rate. The aim of this study was to document information about the mosquito species existing in Nata, Botswana. As a result mosquito specimens were collected in the households by use of pyrethrum sheet collection method and near overgrown vegetation and swimming pool by setting up the h-trap. The specimens were firstly identified using morphological and taxonomic keys as *Anopheles* (41%) or *Culex* (59%). Thereafter, DNA was extracted from the mosquitoes and the 16S rRNA gene was amplified by PCR and then sequenced. The 16S sequences matched with 3 mosquito genera, namely, *Anopheles*, *Culex* and *Aedes*. The results revealed the existence of multiple disease vectors within one territory and this is a first report that identified existence of *Aedes* and *Culex* in Nata village. Further studies must determine the role of these mosquitoes in transmission of malaria parasite (*Plasmodium*) in Nata.

Keywords: Nata, Botswana, Mosquito, *Aedes*, *Culex*, *Anopheles*

4.2 Introduction

The *Anopheles* mosquito, which is responsible for transmission of human malaria parasites comprising of four *Plasmodium* species (*P. falciparum*, *P. vivax*, *P. ovale,* and *P. malariae*) and detection of these species within their hosts is an essential component of vector control (Bass *et al*., 2008). There are as many as 3 800 *Anopheles* species, of which 30 to 40 transmit four different species of human *Plasmodium* parasites that cause malaria (Gimba and Idris, 2014). Proper identification of malaria vectors is very important for the success of an effective vector control programme. Identification of the adult female mosquito is an important aspect in malaria surveillance and control and thus taxonomic keys should be regularly updated and revised for literature update (Gunathilaka, 2017). Moreover, species identification is essential for many biological studies and the identification of morphological characteristics (Erlank *et al*., 2018; Weeraratne *et al*., 2017).

Morphological identification is the conventional ‘gold standard’ method to identify mosquito species based on their external characteristics (Chan *et al*., 2014). Different mosquito species exhibit distinguishable morphological features that are utilized in taxonomic keys such as palps, proboscis, legs, wings and thorax (Chan *et al*., 2014; Williams and Pinto, 2012). However, morphological identification is time consuming and care should be taken to prevent damage to specimens by improper handling (Chan *et al*., 2014).

Taxonomic keys are available for the identification of local malaria vector species, but these keys may differ according to the geographical area and the prevalent vector species (Williams and Pinto, 2012). Moreover, similar morphological characteristics that are shared by members of species complexes make identification a difficult task based on taxonomic keys alone. Most taxonomic keys are limited to adult female mosquitoes and fourth-instar larvae because many of the morphological characteristics are not well developed in early larval stages (Chan *et al*., 2014). Precise identification of mosquito species is very important for an understanding of epidemiological patterns of disease transmission which are usually associated with the abundance level of the vector mosquito (Wilke *et al*., 2016). The latter authors also state that mosquitoes of the genera *Aedes*, *Culex* and *Anopheles* are responsible for the transmission of life-threatening infectious diseases, and it is therefore important to correctly identify and differentiate among these vectors. However, some species such as *Culex* lack distinguishing larval characteristics, hence actual identification can be difficult because members of its complex are morphologically similar (Mathews *et al*., 2017). Morphologically indistinguishable species complexes of *Anopheles*, *Culex* and *Aedes* may have distinct epidemiological roles. Thus correct identification of these species should be done as this will assist in establishing effective vector control initiatives (Wilke *et al*., 2016). It must be noted that, if for any reason the mosquito specimen of interest is damaged, morphological identification may not be possible; hence specimens should be well protected from damage during collection, transportation and storage (Wilke *et al*., 2016).

The morphological identification method uses taxonomic keys and some of the characteristics used are located in the palps, proboscis, legs, wings and thorax (Figure 4.1). Taxonomic keys are available for the identification of different species from both anophelines and culicines and they may differ according to the geographical area and the vector species present (Williams and Pinto, 2012)

4.2.1 External morphology of *Anopheles*

Most, but not all, *Anopheles* mosquitos have spotted wings. This means that dark and pale scales are arranged in small blocks or areas on the veins. The number, length and arrangement of these dark and pale areas differ considerably among different species and provide useful characteristics for species identification. The dorsal and ventral surfaces of the abdomen are about as long as the proboscis and in males, but not in females, they are enlarged (that is clubbed) apically (Awoke and Kassa, 2006; Mohamed, 2017).

4.2.2 External morphology of *Culex*

Frequently, but not always, the thorax, legs and wing veins are covered with scales that are brown in colour. The abdomen is often covered with brown or blackish scales but some whitish scales may occur on most segments. The tip of the abdomen of females is blunt. The claws of all tarsi are simple and those of the hind tarsi are very small. Examination under a microscope shows that all tarsi have a pair of small, fleshy pluvial (Awoke and Kassa, 2006; Mohamed, 2017).

Mosquitoes are one of the most intensely barcoded group of insects because of their importance in disease transmission, and this is especially true for the major Anopheline malaria vectors found across sub-Saharan Africa (Erlank *et al*., 2018; Weeraratne *et al*., 2017). DNA barcoding is a type of molecular characterization that is useful in complementing taxonomy for the identification of mosquito species. This identification method is based on the concept that every species has a unique genetic identity or a DNA barcode that can be used as a genetic marker for species identification (Chan *et al*., 2014). The modern taxonomic system of DNA barcoding has become increasingly popular as it potentially produces results with very high precision and accuracy within a short period of time compared to morphology based taxonomy (Weeraratne *et al*., 2017).

The standard method of using morphological characteristics becomes challenging due to various factors such as phenotypical variations such as genetic makeup. Due to such variations (Karthika *et al*., 2016), mitochondrial DNA has been used and proven to be an important tool in species research as it possesses biological properties for molecular studies. The COI gene is one of the fastest evolving mitochondrial gene identification systems and has often been used in insect genetic studies. The mitochondrial COI gene for molecular identification of mosquito species and other Diptera species was used in various previous studies (Chan *et al*., 2014; Murugan *et al*., 2017; Oliveira *et al*., 2017).

Partial sequences of mitochondrial genes such as Cytochrome c oxidase I (COI), 16S ribosomal RNA (16S rRNA) and 18S ribosomal RNA (18S rRNA) have been used to identify and thus discover new species through DNA barcoding. When fully developed, use of these genes will provide a reliable, cost-effective and accessible solution to the current problem of species identification (Hebert *et al*., 2003; Karthika *et al*., 2016).

DNA barcoding has also proved to be useful where taxonomical identification and morphology based identifications are challenging due to similar or hidden characteristics. DNA barcoding through the use of mitochondrial genes is a molecular approach that uses DNA sequencing to identify and differentiate morphologically similar species. This method requires the use of very small quantities of starting material from partial specimens (Batovska *et al*., 2016). DNA based identification methods require effective DNA extraction from the target specimens, ease in analysing the sequences of the targeted species, and utilizing the sequences to differentiate among the identified species (Murugan *et al*., 2016).

The cytochrome oxidase I (COI) and 16 subunit ribosomal RNA (16S rRNA) genes are the most commonly used mitochondrial sequences in the genetic analysis of insect vectors. These have several advantages which include high resolution in distinguishing among species of the same and different genera, as well as revealing subpopulations in certain species (Debeila, 2010). An example is the use of COI in the molecular identification of mosquitoes in south-eastern Australia in which 29 species, 6 tribes and 12 genera were obtained and, of these, 17 were newly discovered species (Batovska *et al*., 2016).

Cytochrome oxidase is one of the genes that is often used in identification and in insect genetic studies. The COI gene primer sequences as described by Karthika *et al*. (2016) were used in their study to identify fifteen insects that are prevalent in horticultural crops in India. The results of their study showed that COI can be effectively utilized to identify multiple species and can also be an effective method for screening insects and shedding light on their genetic variations in addition to the integration of traditional taxonomy. Mitochondrial ribosomal genes (for example, 16S rRNA and 18S rRNA) can also be used for genetic and phylogenetic studies (Debeila, 2010). The establishment of degree of similarities in different organisms can be done by developing a hierarchy of relationship phylogenetic tree. An example is the review study by Patwardhan *et al*. (2014), who described different molecular markers that may be employed for the purpose of phylogenetic studies. The study also elucidates criteria for choosing suitable markers and steps that lead to the construction of phylogenetic trees.

Figure 4.1 is an illustration of the key features that were investigated by the current study for the identification of the mosquito species that were sampled in Nata village, Botswana.

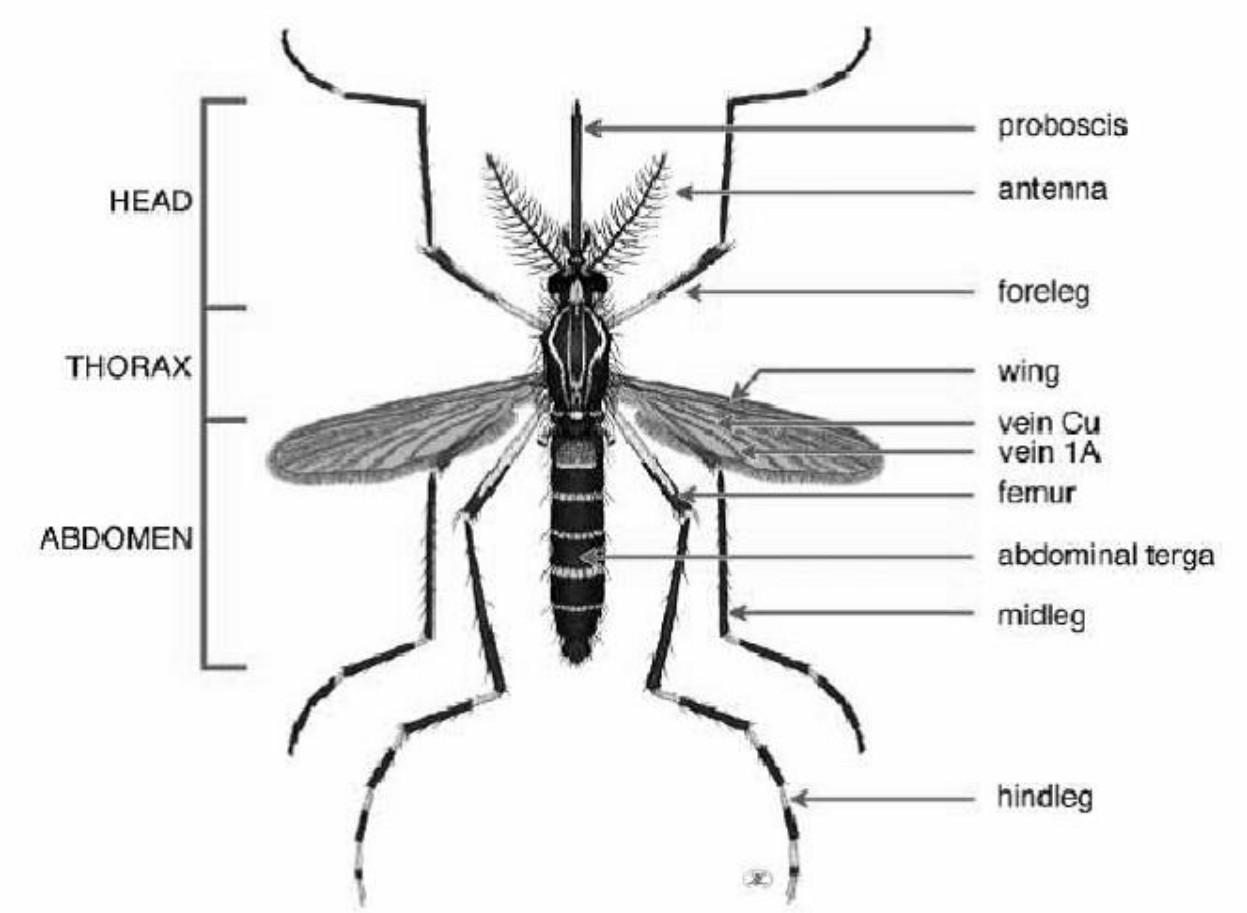

Figure 4.1: Key features used for morphological identification of mosquito species

Source: Rueda, 2004.

4.3 Materials and Methods

4.3.1 Study site and sample collection

Mosquitoes were collected from houses that were generally surrounded by trees. The trap was placed in a place with dense vegetation and near a swimming pool or open water source. Adult mosquitoes were collected from two different locations (the outdoor surroundings and from within houses) in Nata village. The Pyrethrum sheet method and H-trap were used to collect the mosquito specimens.

4.3.2 Pyrethrum sheet method

This method involved the laying of white sheet cloth on the floor and on immovable house furniture. The houses were opened at appropriate times for 30 to 60 minutes to allow the mosquitoes to enter, and then the opened windows and doors were closed and sprayed with a knockdown insecticide (Doom: EPA Registered Name for this product is: Duel flying & crawling insect killer) with active ingredients: D-Phenothrin (pyrethroid) 0.92 g/kg, Prallethrin (ETOC) (pyrethroid) 0.4 g/kg, Imiprothrin (pyrethroid) 0.25 g/kg per 300 ml spray bottle. The insecticide was sprayed according to the manufacturer's instructions. After 15-30 minutes, the white sheet cloth was carefully taken out of the house to collect the knocked down mosquitoes.

4.3.3 H-trap

The trap (Figure 4.2) was placed in a shade near a swimming pool and a solid waste receptacle was set up. The trap was monitored for five days and acetone was used as an attractant to the trap.

Figure 4.2: H-trap

Source: Photograph by O.I. Nkaiwa (author) on the 21st November 2017

4.4 Sample Preservation

All the collected adult mosquitoes were kept in a labelled sampling bottle, preserved with 70% ethanol, and stored in 4^{o}C temperature fridge until analysis.

4.5 Morphological Identification of the Mosquito Specimens

Adult *Anopheles* mosquitoes were identified by external morphology using dichotomous keys (Gillies and Coetzee, 1987; Coetzee *et al*., 2013) and information provided by previous studies. The mosquitoes were examined under a microscope using adult morphological identification key for *Anopheles* spp. (Gillies and Coetzee, 1987; Coetzee *et al*., 2013) and *Culex* spp. (Azari-Hamidian and Harbach, 2009). The useful morphological characteristics to distinguish between the Anopheline and Culicine species were the wings, legs, thorax, abdomen, head and mouthparts. The following studies were also consulted for the morphological identification of the specimens: Atting and Akpan (2016); Gunathilaka (2017); Chirebvu *et al*. (2014); Tawe *et al*. (2017); Erlank *et al*. (2018); Amerasinghe *et al*. (2002); Pombi *et al*. (2015); Chan *et al*. (2014); Gimba and Idris (2014); Kaindoa *et al*. (2017); and Rattanarithikul *et al*. (2006).

4.6 Molecular Identification of the Mosquito Specimens

4.6.1 Extraction of Mosquito DNA

Total DNA was extracted from an intact mosquito (excluding wings and legs) using the DNeasy blood and tissue kit (Qiagen, Germany) according to the manufacturer's instructions (the protocol is presented in Appendix II).

A Nano drop spectrophotometer (Thermo Fischer, USA) was used to confirm the presence of the DNA before storage at 20°C until used.

4.6.2 PCR for the detection of the DNA of the *Plasmodium* species from the specimens

PCR was conducted to detect the species of the four malaria parasites that infect humans, namely *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium ovale and Plasmodium vivax* from the extracted genomic DNA of the mosquitoes. PCR primers targeting the 18S rRNA gene were used as presented in Table 4.1.

Table 4.1*: Plasmodium* primers used in this study

Type of primer	Primer name	Sequence (5' ---- 3')	Reference
Universal	P1	ACGATCAGATACCGTCGTAATCTT	Han *et al*., 2007
Universal	P2	GAACCCAAAGACTTTGATTTCTCAT	Han *et al*., 2007
Pfa-specific	Pfa*	CAATCTAAAAGTCACCTCGAAAGATG	Han *et al*., 2007
Pvi-specific	Pvi*	CAATCTAAGAATAAACTCCGAAGAGAAA	Han *et al*., 2007
Pov-specific	Pov*	ACTGAAGGAAGCAATCTAAGAAATTT	Han *et al*., 2007
Pma-specific	Pma*	GGAAGCTATCTAAAAGAAACACTCATAT	Han *et al*., 2007

Pfa*: *Plasmodium falciparum*; Pvi*: *P. vivax*; Pov*: *P. ovale*; Pma*: *P. malariae*

The universal primers for the SSU rRNA gene of all *Plasmodium* species (P1 and P2) and the specific primers of *Plasmodium falciparum* (Pfa) were used in the identification of the *Plasmodium* species from the extracted DNA.

In the first PCR, only universal primers were used to first detect the *Plasmodium* parasite in the mosquito DNA prior to detecting the specific *Plasmodium* species. The PCR master mix was prepared as follows: 39.75 µl of double distilled water, 5 µl of 10X standard Taq reaction buffer, 2 µl primer mix (P1 and P2), 1 µl dNTPs, 0.25 µl *Taq* DNA polymerase, and 2 µl extracted DNA to the final volume of 50 µl.

The PCR conditions were as follows: initial denaturation steps at 95°C for 5 minutes; then the samples were submitted to 35 cycles of denaturation at 95°C for 30 seconds. Annealing of the primers occurred at 60°C for 60 seconds and extension at 68°C for 1 minute. A final cycle extension of 5 minutes at 68°C was included, followed by final hold at 4°C∞.

4.6.2 PCR amplification of mosquito DNA (CO1, 16S rRNA, 18S rRNA)

The PCR for the molecular identification of the mosquito specimens was performed targeting 710 bp CO1, 300 bp 16S rRNA, and 780 bp 18S rRNA genes using primers as listed in Table 4.2. Each reaction mixture with a total volume of 25 µl consisted of 12.5 µl AmpliTaq Gold 360 Master Mix (Applied Biosystems, USA), 1 µl of 10 µM forward primer, 1 µl of each of 10 µM of reverse and forward primers, 2 µl template DNA, and 8.5 µl double distilled water. Furthermore, double distilled water was used as the negative control and *Rhipicephalus sanguineus* (brown dog tick) DNA was used as a positive control.

PCR conditions for CO1 were as follows: Initial denaturation at 95°C for 10 minutes, 35 cycles of denaturation at 95°C for 30 seconds, annealing at 47°C for 30 seconds, and extension at 72°C for 60 seconds, followed by a final extension at 72°C for 7 minutes and final hold at 4°C, using the ProFlex PCR System (Thermo Fisher Scientific, USA).

For 16S rRNA, the PCR conditions were: Initial denaturation at 95°C for 10 minutes, 35 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 60 seconds, followed by a final extension at 72°C for 7 minutes and final hold at 4°C.

For 18S rRNA the PCR conditions were: Activation at 95ºC for 10 minutes, followed by 35 cycles at 95ºC for 30 seconds, annealing at 52ºC for 30 seconds, extension at 72ºC for 1 minute, final extension at 72ºC for 7 minutes, and final hold at 4°C.

Table 4.2: PCR primers used to amplify COI, 16S rRNA and 18S rRNA genes

Target gene	Primer sequence (5'- 3')	Fragment size (bp)	Annealing temperature (°C)	Reference
CO1	LCO1490: 5'- GGTCAACAAATCATAAAGA TATTG G -3'	710	47	Licari *et al.,* 2017
	HCO2198: 5'- TAAACTTCAGGGTGACCAA AAAATCA -3'			
16S rRNA	N1-J-12585: 5'- GGTCCCTTACGAATTTGA ATA TATCCT-3'	300	50	Wohlford *et al*., 1999
	LR-N-12866: 5'- ACA TGA TCT GAG TTC AA CCG G-3'			
18S rRNA	18S-F: 5'-CATTAAATCAGTTATGGTTCC-3'	780	52	Lv *et al*., 2014
	18S-R: 5'-CGCCGCAATACGAATGC-3'			

4.6.3 Agarose gel electrophoresis

Amplified DNA of all samples was confirmed by gel electrophoresis of 1% (w/v) stained with ethidium bromide and size fractioned using a 100 bp DNA ladder (New England Biolabs, USA). The stained agarose gel was poured into the trough and the comb placed at the edge until it solidified. Then 2 µl of the loading dye was mixed with PCR amplicons and added in their respective wells, one by one.

The gel was visualized under UV light using the ENDURO GDS Gel Documentation System (Labnet International Inc., USA). Amplified DNA products were sent to Inqaba Biotechnological Industries (Pty) Ltd (Pretoria, ZA) for sequencing.

4.6.4 Phylogenetic analysis

The gene sequences (CO1, 16S rRNA and 18S rRNA) that were retrieved from Inqaba Biotechnological Industries were viewed and edited using Trace Editor on MEGA 7 (Ma *et al.*, 2015). Post editing, all the sequences were subjected to the nucleotide Basic Local Alignment Search Tool (BLASTn) to confirm if they matched their respective reference sequences on the NCBI database. Unfortunately, only 16S rRNA could be used for further analysis as no significant matches could be found on the NCBI database for CO1 and 18S rRNA sequences. For 16S rRNA phylogenetic analysis, reference sequences were downloaded from NCBI GenBank and they were all aligned by Clustal W with default parameter settings and thereafter truncated to even sizes. The best model was selected based on the lowest Bayesian Information Criterion (BIC) score on MEGA 7. Subsequently, the maximum likelihood tree with 1000 bootstrap support value was selected and Tamura 3-parameter with five Gamma distributions was selected as the best substitution model.

4.7 Results and Discussion

4.7.1 Morphological identification of the mosquito specimens

In total, 55 mosquitoes were collected from Nata village within a period of 10 days and 27 were used first for morphological identification. Through the use of Wild Heerbrugg (Switzerland) microscope and a smartphone (Huawei) for photographs, various physical characteristics were observed (Table 4.3). The photographs had to be subjected to editing by downloading to a laptop and editing with paint 3D photo software for better photographic effects and for some of the characteristics to be visible (Appendix I, Plate I-VI).

TABLE 4.3: Morphological characteristics used to identify mosquito specimens

Features	Characteristics
Colour	Brown
Legs	Spotted/speckled
Abdomen	Brown or blackish scales
Proboscis/palps	Black colour at the ends
Wings	Dark/black wing veins

With reference to the literature, the majority of the mosquito samples used for morphological identification could be positively identified through morphological and taxonomic keys (Figure 4.3). The findings were: *Anopheles* (41%) or *Culex* (59%) with physical similarities (Atting and Akpan, 2016; Gunathilaka, 2017; Chirebvu *et al*., 2014;

Tawe *et al*., 2017; Erlank *et al*., 2018; Amerasinghe *et al*., 2002; Pombi *et al*., 2015; Chan *et al*., 2014; Gimba and Idris, 2014; Kaindoa *et al*., 2017).

In order to solve the problem of morphological similarities in the specimens, molecular identification (DNA barcoding) for confirmation of morphological results was carried out as had been done in an earlier study by Chan *et al*. (2014). Plates I-VI (see Appendices) illustrates similar characteristics and taxonomic keys that were visible in all the mosquito specimens used for morphological identification.

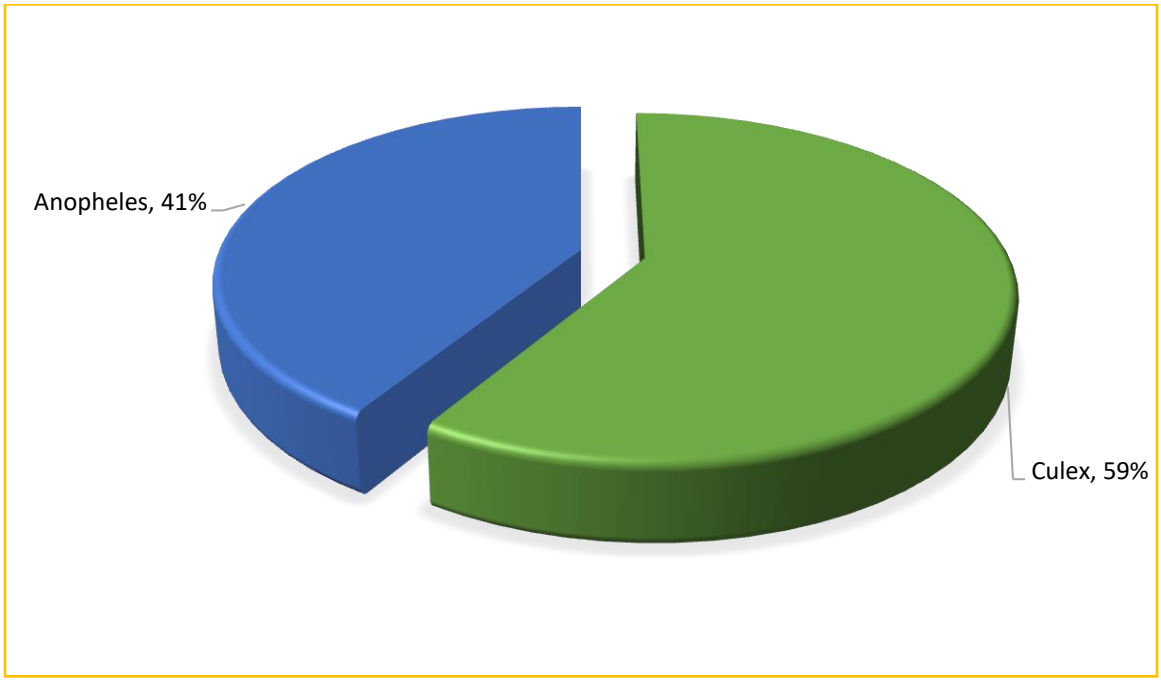

Figure 4.3: Pie-chart depicting the morphologically identified species

Previous study findings in Botswana (Tawe *et al*., 2017) revealed that *Anopheles arabiensis* was widely spread in all seven districts where the study was conducted. The latter study also found that, in the same district where Nata village is located, the prevalence of this mosquito species was 100%.

4.8 Molecular Identification of Mosquitoes

Of the 55 mosquito specimens, only 28 were used for molecular identification.

4.8.1 Polymerase chain reaction for *Plasmodium* species identification

No *Plasmodium* species were detected by PCR using the 18S universal primers (Figure 4.4). Using mosquito DNA for *Plasmodium* species detection, none were amplified, which means that the mosquitoes were not infected with the *Plasmodium* parasite.

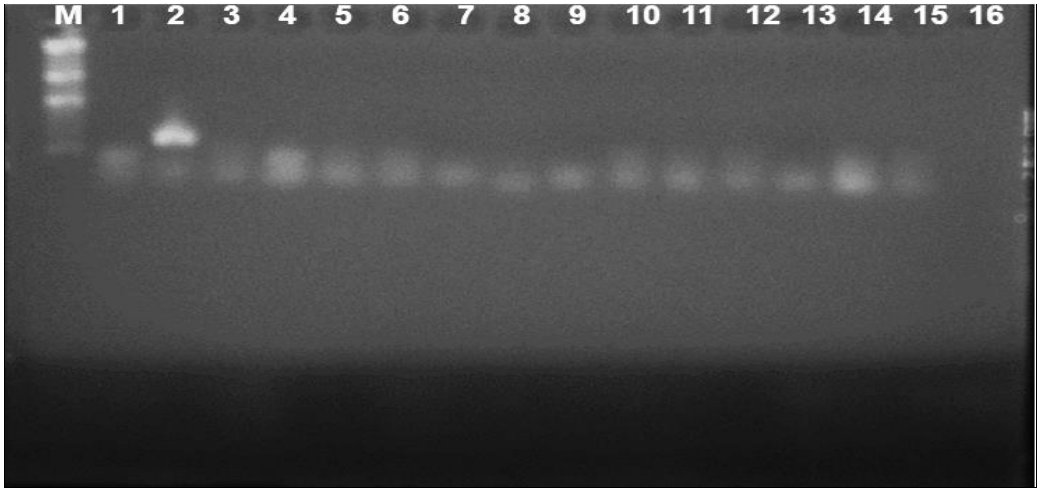

Figure 4.4: Agarose gel electrophoresis of mosquito DNA PCR for *Plasmodium* species identification. M: DNA ladder (100bp), Lane 1: negative control (DDH_2O) and 2: positive control (universal *Plasmodium* primer). Mosquito DNA samples (Lanes 3 -16).

4.8.2 PCR for CO1, 16S rRNA, and 18S rRNA amplification

The COI (Figure 4.5), 16S rRNA (Figure 4.6) and 18S rRNA (Figure 4.7) genes were successfully amplified from mosquito DNA samples. The length of CO1, 16S rRNA and 18S rRNA sequences were 710, 300 and 780 bp respectively.

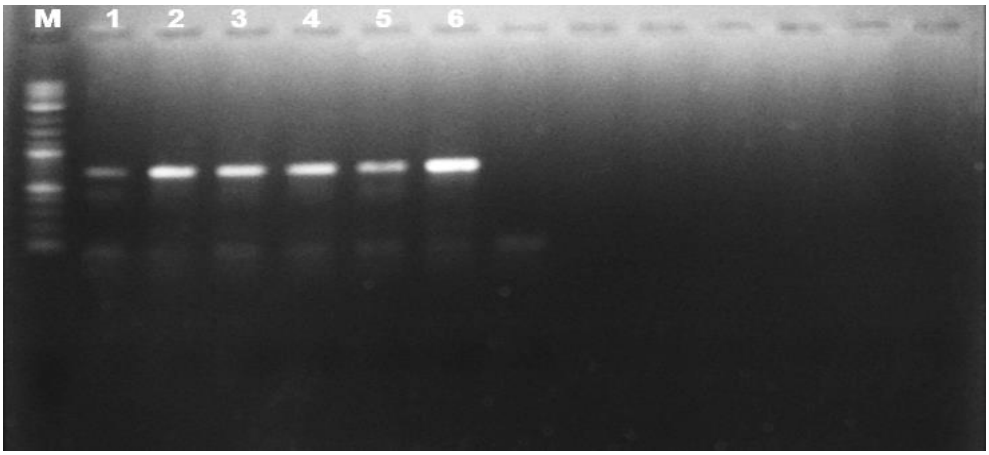

Figure 4.5: Agarose gel electrophoresis of mosquito DNA PCR for CO1 gene amplification. M: DNA ladder (100bp), Lane 1: negative control (DDH_2O) and 6: positive control (*Rhipicephalus sanguineus* (brown dog tick) DNA. Mosquito DNA samples (Lane 2 - 5).

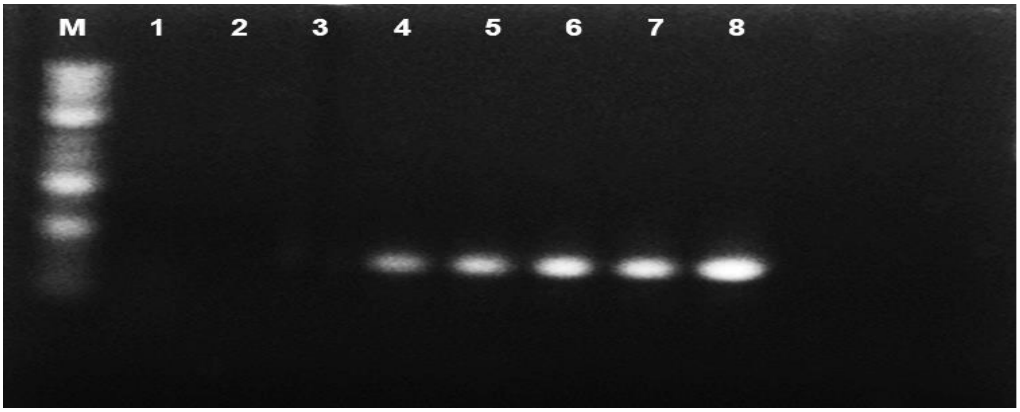

Figure 4.6: Agarose gel electrophoresis of mosquito DNA PCR for 16S rRNA gene amplification. M: DNA ladder (100bp), Lane 1: negative control (DDH_2O) and 8: positive control (*Rhipicephalus sanguineus* (brown dog tick) DNA. Mosquito DNA samples (Lane 4 - 7). Lane 2 and 3 did not amplify.

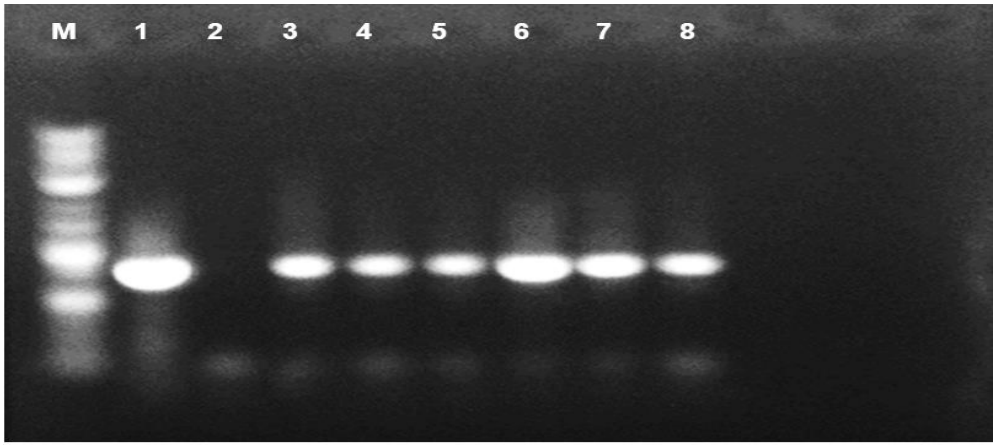

Figure 4.7: Agarose gel electrophoresis of mosquito DNA PCR for 18S rRNA gene amplification. M: DNA ladder (100bp), Lane 1: positive control (*Rhipicephalus sanguineus* (brown dog tick) DNA and 2: negative control (DDH_2O). Mosquito DNA samples (Lane 3 – 8).

4.8.3 Phylogenetic analysis

4.8.3.1 The 16S gene

The alignment of the DNA sequences of the mosquito species that were analysed resulted in a total of 172 positions, including gaps. The average length of the sequenced 16S gene for mosquito fragments was 295 bp with Maximum Likelihood (ML) phylogenetic analysis were from species of *Culex*, *Aedes* and *Anopheles* with *Sabethes undosus* used as an outgroup (Figure 4.8).

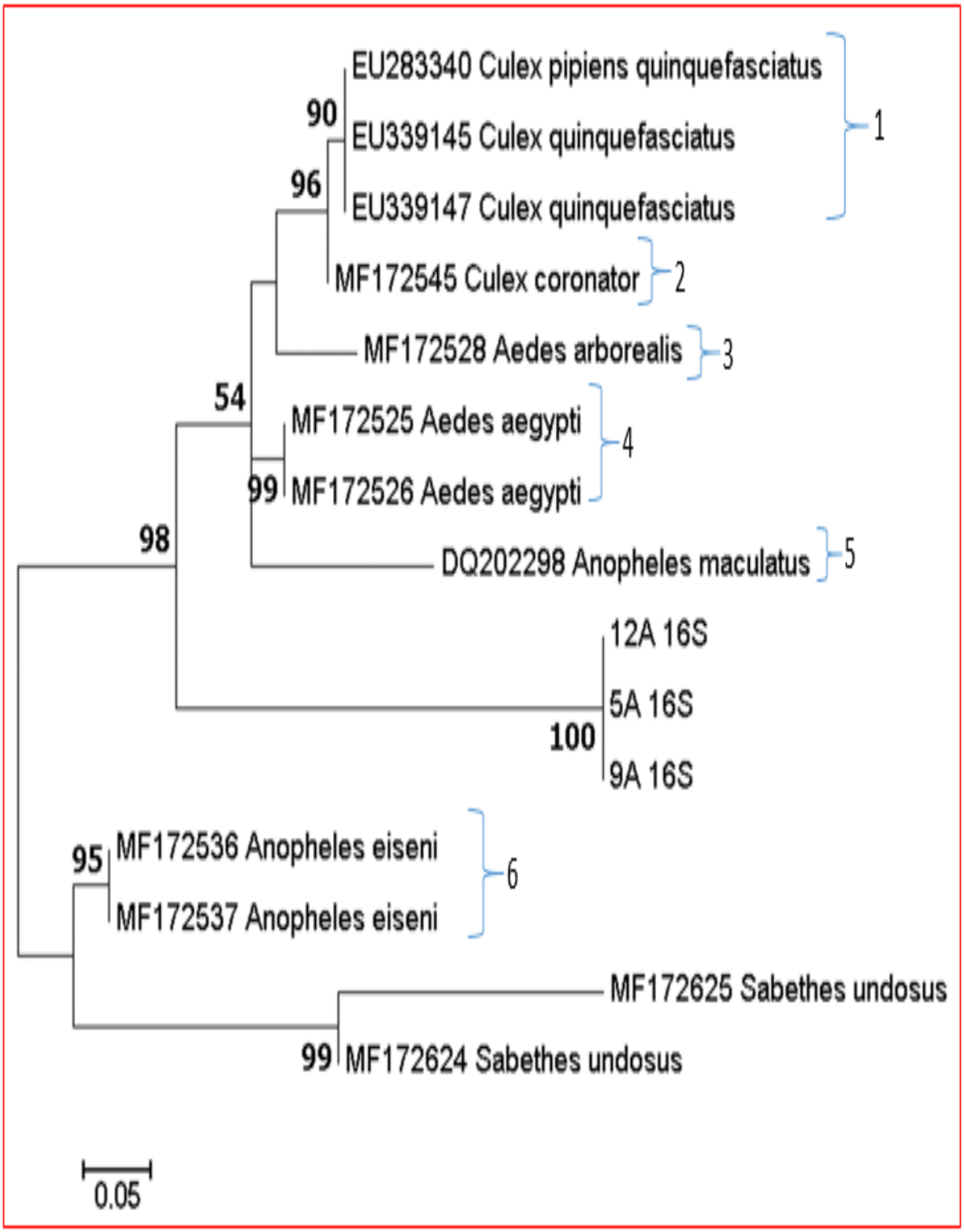

Figure 4.8: Phylogenetic analysis of the 16S rRNA gene of mosquitoes using the Maximum Likelihood method (1- 6 represent species generated)

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model (Nei and Kumar, 2000). The tree with the highest log likelihood (-793.37) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value.

A discrete Gamma distribution was used to model evolutionary rate differences among sites (2 categories +G; parameter = 1.1532). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analyses involved 15 nucleotide sequences (Appendix II). Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 172 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2016).

The findings generated by the phylogenetic tree shows that there is possibility of different species of mosquitoes within one location.

4.9 Conclusions

4.9.1 Morphological analyses

By means of morphological analyses for the identification of a majority of the samples, similar external morphologies of *Anopheles* (41%) and *Culex* (59%) species were revealed.

According to Tawe *et al*. (2017), *Anopheles arabiensis* was the most prevalent and widespread mosquito species in all the districts that were surveyed in Botswana. The latter study revealed a large variety of species at different sites, but *Anopheles arabiensis* at 58.9% had the highest relative frequency in five of the seven districts where the study was conducted. Tawe *et al.* (2017) also found that Tutume district, where the current study was conducted, had a 100% prevalence of the *An. arabiensis* species, which was also at first confirmed by the current study through morphological examinations. However, the morphological findings of this study could not state beyond any doubt that the samples comprised 100% of the *Anopheles* species, because different species of mosquitoes that are vectors of different mammalian diseases may be morphologically identical and may thus require the expertise of experienced taxonomists for identification. Morphological identification can be complemented by DNA barcoding as an alternative tool to identify species. An example of this is a study that was conducted by Weeraratne *et al*., (2018) where DNA barcoding was used when the traditional morphological identification failed due to indistinguishable characteristics of damaged specimens and the presence of subspecies. Many mosquito species are morphologically indistinguishable and form a cryptic species complex which requires a molecular approach as the only effective tool for resolving their identification, and this is done using the DNA barcoding approach (Rozo-Lopez and Mengual, 2015). In the current study DNA barcoding was also carried out to complement the morphological results.

Anopheles arabiensis belongs to the *An. gambiae* species complex and is one of the most dominant malaria vector species (Sinka *et al*., 2010). It is also one of the mosquito species that can survive in dry savannah environments as its larvae are able to survive in temporary, small clear and shallow fresh water pools (Drake and Beier, 2014; Sinka *et al*., 2010). This was found by Tawe *et al*. (2017), who revealed a 100% prevalence of *Anopheles arabiensis* in the district that Nata village is found, a location that is characterized by dry savannah-type vegetation. They discovered that most of the breeding sites were small puddles created by receding floods, animal hoof prints, small depressions along waterways fed by rain water, and small puddles present all over the previously flooded crop fields and surrounding areas. Finding these larvae proved that these conditions are conducive to *Anopheles arabiensis.* Furthermore, Chirebvu and Chimbari (2014) argue that *An. arabiensis* tends to predominate in arid savannah of sub-Saharan Africa, which is corroborated by Davies *et al.* (2016) who showed that these larvae developed, at three temperature treatments (20–30°C, 25°C and 18–35°C). The above literature on climate and temperatures that characterize the surrounding led to an assumption that the *An. arabiensis* species had high probabilities to exist in the Nata location.

4.9.2 *Plasmodium* species detection

The universal *Plasmodium* primer was not successfully amplified from mosquito DNA in the gel electrophoresis. The results thus indicated that the mosquito specimens were not infected with the *Plasmodium* parasite, hence no further PCR investigations were done with species-specific *Plasmodium* primers.

A reason for not detecting mosquitoes infected with the *Plasmodium* parasite could be that the specimens that were collected were not *Plasmodium* parasite carrying mosquito species of the genus *Anopheles*.

This assumption was confirmed by the phylogenetic analysis that proved beyond any doubt that the mosquito samples that had been collected were species that are not able to be infected by the malaria causing parasite, as they were of the *Culex* species. A study by Molina-Cruz *et al*. (2013) to determine whether culicine mosquitoes could transmit malaria stated as a fact that *Plasmodium* species are not transmitted by culicine, but that they are main vectors for other human pathogens and they are widely spread and present in areas where malaria is endemic.

It has been demonstrated that several mammalian-infectious *Plasmodium* species can reach advanced developmental stages in some culicine mosquitoes, but there is no evidence that culicines transmit malaria to humans (Molina-Cruz *et al*., 2013). The current research report thus merely assumes that the *Culex* species identified in Nata village could be a possible contributing vector to malaria transmission, but it is urged that this is conjecture and thus calls for further and broader studies.

4.9.3 Phylogenetic analysis

The maximum likelihood tree constructed using the 16S sequences formed six supported clades and three mosquito genera were generated, namely *Anopheles*, *Culex* and *Aedes*. The genera generated are vectors of different diseases and breed in different habitats.

For instance, some *Anopheles* species breed in small, open, sunlit, fresh water bodies, and others breed in water bodies with emergent vegetation such as swamps and rice fields and most of the *Culex* species breed in polluted water habitats such as pit latrines, soak pits, cesspits and open sewage systems (Emidi, Kisinza, Stanley *et al*., 2017).

Although a recent study by Tawe *et al*. (2017) revealed a 100% distribution of *Anopheles arabiensis* in the same district as our study, this study also, through the use of 16S marker, revealed other species of *Anopheles*, *Culex* and *Aedes*. However, culicine mosquitoes (e.g., *Aedes* spp. and *Culex* spp.), which are common worldwide, are also abundantly present in areas where malaria is endemic. They feed on human blood and are, just like anophelines, important vectors for human pathogens. They also transmit filarial worms and arboviruses such as Yellow Fever, Dengue, and the West Nile virus (Molina-Cruz *et al*., 2013).

4.9.3.1 *Culex species* (*C. quinquefasciatus* and *C. Coronator*)

Using 16S marker, the study discovered that the *Culex* species occurred in Nata village. Although it is not a malaria transmitting vector, the *Culex* species is widely prevalent in malaria endemic areas and according to Emidi, Kisinza, Stanley *et al*. (2017), it is regarded as a 'nuisance mosquito'. Botswana as a landlocked country is characterized by hot, dry summers with rain during October to December and January to March, while the rest of the months are hot and dry.

A study by Rueda *et al*. (1990) on the temperature-dependent development and survival rates of *C. quinquefasciatus* revealed that larvae survival to adult stage occurred up to a temperature of 34°C, but the current study discovered that this species in Nata village survived in temperatures up to 40°C. The mosquito traps used in the current study were placed near swimming pools, near waste receptacles that had rotting garden waste, and near overgrown vegetation surrounding a soakaway pit. These are common habitats for *Culex* and this is probably the reason why mosquitoes from this species were discovered in Nata village.

Also the study by Emidi, Kisinza, Stanley *et al*. (2017) discovered the *Culex* species in similar habitats, and they stated that the breeding sites of these mosquitoes are sometimes contaminated with pollutants from various sources such as sewage and fertilizers from agricultural contexts. Members of the *Culex* species breed in a large variety of still waters ranging from artificial containers and catchment basins of drainage systems to large bodies of permanent water. The most common species, *C. quinquefasciatus,* is a major nuisance and vector of filariasis. It breeds especially in water polluted with organic material such as refuse and excreta or rotting plants. Examples of such breeding sites are soakaway pits, septic tanks, pit latrines, blocked drains, canals, and abandoned wells. In many developing countries, *C. quinquefasciatus* is common in rapidly expanding urban areas where drainage and sanitation are inadequate (Rozendaal, 1997).

C. quinquefasciatus is a markedly domestic species. The adult females bite people and animals throughout the night, both indoors and outdoors. During the day they are inactive and are often found resting in dark corners of rooms, shelters and culverts. They also rest outdoors on vegetation and in holes in trees in forested areas (Rozendaal, 1997; McClure *et al*. 2018). Similar to other culicines, *C. coronator* is an invader species that is most likely to be discovered in malaria endemic areas. A study by Yee and Skiff (2014) revealed that *C. coronator* and *C. quinquefasc*iatus exhibited similar levels of larval performance, at least under the conditions used and the density tested. The *C. coronator* nominally uses small natural aquatic habitats for development. Its use of containers (e.g. tyres) makes it potentially important and it is suggested that the performance of *C. coronator* is similar to that of *Culex quinquefasciatus* (Yee and Skiff, 2014).

4.9.3.2 The *Aedes* species (*Ae. aegypti* and *Ae. arborealis*)

Aedes mosquitoes occur around the world and there are over 950 species. They can cause a serious biting nuisance to people and animals, both in the tropics and in cooler climates. In tropical countries, *Ae. aegypti* is an important vector of Dengue, Dengue Haemorrhagic Fever, Yellow Fever, and other viral diseases. *Ae. aegypti* mainly breeds in domestic environments where its preferred habitats are water storage tanks and jars inside and outside houses, in roof gutters, leaf axils, bamboo stumps and temporary containers such as jars, drums, used car tyres, tin cans, bottles, and plant pots. All these habitats typically contain relatively clean water (Rozendaal, 1997).

Furthermore, Noden *et al*. (2014) conducted a study on risk assessment in southern Africa of flavivirus transmission in Namibia through a survey of tyre sites in Windhoek. This study revealed that the presence of *Ae. aegypti* is highly likely in Botswana and urged further research to corroborate this. *Aedes* mosquitoes bite mainly in the morning or evening. Most species bite and rest outdoors but in tropical towns *Ae. aegypti* breeds, feeds and rests in and around houses.

4.9.3.3 The *Anopheles* species (*An. maculatus* and *An. eiseni*)

This study revealed the presence of *An. maculatus and An. Eiseni* in the study area. Members of the *An. maculatus* group are typically found in or near hilly and mountainous areas. These larvae have been collected in a diverse number of permanent or semi-permanent bodies of clean water that are often exposed to direct sunlight including ponds, lakes, swamps, ditches, wells, different types of pools (grassy, sandy, ground, flood, stream), margins along small, slow-flowing streams, gravel pits along stream margins, seepages, springs, rice fields, and occasionally tree holes and bamboo stumps (Sinka *et al*., 2011). Members of the *An. maculatus* group are variously involved in malaria transmission. However, the vector role of each species is not precisely known and their vectorial capacity appears to vary depending on geographical location. The *An. maculatus* complex includes important malaria vectors distributed from the Indian subcontinent through Southeast Asia to Taiwan (Sinka *et al*., 2014).

Even though a previous study in the same district where Nata village is situated found that only *An. arabiensis* is widely prevalent (Tawe *et al*., 2017), the present study revealed the presence of *An. maculatus* as well. Studies by Sinka *et al*. (2012) devised a global map of dominant malaria vectors and revealed that this species is widely spread in Asia. This new acquired information based on the current study led to the assumption that this is one of the contributing malaria vectors in Nata village. It is hoped that similar data will become increasingly available as the importance of correctly and fully identifying these species has become more widely accepted, thus allowing for updated and detailed species-specific maps to be produced in the future.

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CHAPTER 5

GENERAL DISCUSSION, CONCLUSION & RECOMMENDATIONS

5.1 Discussion

Substantial advancements have been made in malaria control particularly in sub-Saharan Africa since 2000, but there are strong indications that the active involvement of communities in malaria control and elimination programmes has not been fully achieved. The overall impact of good malaria control is a reduction in malaria morbidity and mortality (Snow and Marsh, 2010). Community engagement is critical for the uptake of prevention, diagnosis and treatment, while concurrently shedding light on how knowledge, beliefs and practices affect the acceptability of interventions by the community (Van den Berg *et al*., 2018). These community engagements could be in the form of community workshops on malaria facilitated by trained health facilitators. Education plays an important role in malaria knowledge and the adoption of stringent control measures (Padonou *et al*., 2018), whereas substantial knowledge of malaria reinforces the capacity of the community to influence transmission intensity through positive attitudes and behaviour towards malaria. Moreover, heightened community operations and collaboration with health facilities and community health workers further outline their important role in malaria prevention and control (Shimaponda-Mataa *et al*., 2015). In light of the above, the current study was conducted to determine the extent of malaria knowledge and malaria control methods among randomly selected participants in Nata village, Botswana.

The literature revealed that long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) are commonly used together in the same households to improve malaria control despite inconsistent evidence on whether such combinations actually offer better

protection than nets alone or IRS alone (Okumu *et al*., 2013). WHO recommends that malaria endemic countries protect all those at risk of malaria with long-lasting insecticide-treated nets and, where appropriate, indoor residual spraying (Chaccour *et al*., 2018). Research has shown that the contribution of IRS and LLINs has been immense in recent reductions of the malaria burden even though their effectiveness is threatened by widespread resistance to the insecticides delivered through these interventions, especially pyrethroids (Ngufor *et al*., 2017). Due to high rates of new cases and deaths that occurred in sub-Saharan Africa in 2015, WHO recommended that all persons at risk of contracting malaria should be provided with at least one vector control intervention: either the use of long-lasting insecticidal nets or indoor residual spraying. However, due to the high cost of these interventions, most malaria endemic countries face the dilemma of resource allocation and prioritization to support vector control (Lee *et al*., 2017).

5.2 Summative Remarks

5.2.1 Chapter 3

Education, community involvement and engagement are vital in malaria control. This study found that 100% of the 109 participants demonstrated sufficient knowledge of use of available malaria control interventions that are implemented in the village through government initiatives.

Despite fair knowledge of malaria control intervention such as LLINs and IRS; there remains a need to improve the availability and accessibility of these interventions through education, the dissemination of information, and sustainable support

interventions provided by the government. For education campaigns to be effective, media and community workshops should be conducted and rolled out to rural communities. The current study revealed that only 12% of the participants preferred the use of both LLINs and IRS for sustainable protection against malaria. Only through education and information dissemination will the rest of the community come to acknowledge the value of the combined use of these measures. It is inarguable that the success and sustainability of malaria control interventions are dependent on the positive attitudes and active participation of the entire community as encouraged by WHO.

5.2.2 Chapter 4

5.2.2.1 Morphological identification

Knowledge of the mosquito species and its impact on human health is essential for effective control of mosquito prevalence and malaria transmission. The aim of this chapter was to identify species from a batch collected from a malaria endemic area (Nata village in Botswana). *Anopheles* (41%) and *Culex* (59%) species were identified. By means of an entomological investigation, the physical characteristics of *Anopheles* were identified in the specimens, which are in many respects the same as those of *Culex.* As this was a new discovery of the latter species in this area, further research in the prevalence of this species in this area is vital.

Many *Anopheles* species feed on both humans and animals. Some species feed mostly on animals while others feed almost entirely on humans, thus these are highly dangerous as vectors of malaria (Rozendaal, 1997). *Culex quinquefasciatus* and many

other *Culex* species bite humans and other hosts. Some species, such as *Culex quinquefasciatus*, commonly rest indoors both before and after feeding, but they also shelter in outdoor resting places (Mohamed, 2017).

5.2.2.2 Molecular identification

Several authors (Chan *et al*., 2014 and Weeraratne, Surendran, Karunaratne *et al*., 2018) have shown that molecular analysis through DNA barcoding or molecular characterization is an efficient method for species identification because it produces results with high precision within a short period of time. Based on molecular identification, no *Plasmodium* species (carriers of malaria) were detected by PCR using the 18S universal primer. The mosquito specimens that had been collected were therefore not infected with the *Plasmodium* parasite and it was therefore assumed that it might be a non-malaria vector because previous studies have shown that other species can be found within malaria endemic areas.

Due to their rapid rate of evolution and similar morphological characteristics, mitochondrial genes are believed to give better resolutions in population genetic studies than nuclear DNA genes. The objective of this phase of the study was to identify the mosquito species collected in Nata and to complement and verify morphological identification results. Mitochondrial genes (16S rRNA, 18S rRNA and CO1) were used to run PCR on mosquito DNA and all successfully amplified. However, the amplified DNA products were sent to Inqaba Biotech (Pretoria, ZA) for sequencing and

unfortunately 16S rRNA had to be exclusively used for phylogenetic analysis as the CO1 and 18S rRNA sequences found no significant matches on the NCBI database.

Three genera of mosquitoes, namely *Anopheles*, *Aedes* and *Culex*, were identified in Nata village in this study. Seven mosquito species namely *C. quinquefasciatus*, *C. pipiens quinquefasciatus*, *C. coronator*, *Ae. aegypti*, *Ae. arborealis, An. maculatus*, *and An. eiseni* were also identified in the phylogenetic tree revealing the existence of multiple disease vectors within one territory.

5.3 Conclusion

The study achieved its objectives:

(I) The knowledge of and preferences pertaining to malaria control among selected villagers in Nata village in Botswana were determined and their intervention strategies could be assessed;

(II) Morphological and molecular identification of mosquito species in Nata village were conducted, revealing the existence of a multiple number of mosquito species and the absence of *Plasmodium* infection in the specimens.

Based on the findings, the following conclusions were drawn:

(I) In terms of malaria interventions, it was found that the community of Nata had a fair knowledge of various prevention measures, but they preferred using LLINs rather than other interventions to curb the threat of contracting malaria. However, the demonstrated lack of a combination of measures to curb infection poses a threat to this community as one measure alone may not be effective and sustainable, particularly as the effectiveness of the nets wears off after a time.

(II) Morphological analyses detected the presence of two genera of mosquitoes (*Anopheles* and *Culex*). According to its physical characteristics, *Culex* showed a higher prevalence than *Anopheles*. This was unexpected findings that demonstrated the value of in-depth morphological analyses and possibly follow up studies.

(III) By complementing the morphological analyses that had been conducted, molecular identification revealed seven species of mosquitoes with additional genera of *Anopheles*.

(IV) The presence of *C. quinquefasciatus, C. pipiens quinquefasciatus, C. coronator, Ae. aegypti, Ae. arborealis, An. maculatus*, and *An. eiseni* as some of the mosquito species that exist in Nata village, Botswana has been revealed for the first time by this study.

(V) However, the *Plasmodium* parasite was not detected in the mosquito specimens under study. A plausible explanation could be that the specimens could not have been infected at the time of collection, or that the number of

mosquitoes that were collected had somehow been directly affected by the sampling time.

5.4 Recommendations

It is essential that the results of the current study be disseminated appropriately to policy makers to inform them of ordinary citizens' (villagers') preferred method(s) for malaria prevention. This knowledge may be used advantageously to promote both the discussed and other effective malaria prevention methods. The results are also informative regarding existing mosquito species distribution in Nata village, Botswana. Although the findings of this study may not be generalized, they may be used to inform similar studies as well as potential intervention methods in similar regions and villages.

(I) Community education through workshops should be intensified to promote attitudes that are based on knowledge and information and that encourage participation in studies that need community involvement.

(II) Different mosquito sampling techniques should be used for high yields of specimens.

(III) A set of strategies should be formulated to promote the implementation of local prevention and control measures aimed at minimizing the exposure, occurrence and spread of malaria and to promote positive attitudes and best protective practices.

(IV) Only 109 participants consented to take part in the study over a period of 10 days. Future research should be conducted over at least 31 days to capture more participants and maximize results and outcomes. The study participants' ages ranged from 17 years to 80 years. It was assumed that it would be difficult to reach participants under the age of 17 years in secondary schools. Future studies should include school children, particularly at secondary school level, to document the level of knowledge and education of this portion of the community about malaria control strategies. Only when the leaders and parents of the future are highly informed of the threat of malaria and measures to combat it, these measures will become more successful and will lead to eradication of malaria in endemic regions.

(V) This is the first descriptive cross-sectional survey study on the knowledge, preferences and views of the residents of Nata village in Botswana regarding malaria control methods. Although the findings of this study are not generalizable, they could be used by health authorities to evaluate all methods of malaria control. This may result in the development of strategies that will be effective in the eradication of malaria in the study area, or even in other similar malaria endemic areas.

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APPENDICES

Appendix I: Plates I-VI of the morphological characteristics of the collected mosquito specimen from Nata village

Plate I: Abdomen

Plate II: Proboscis

Plate III: Foreleg

Plate IV: Wing 1 showing black veins

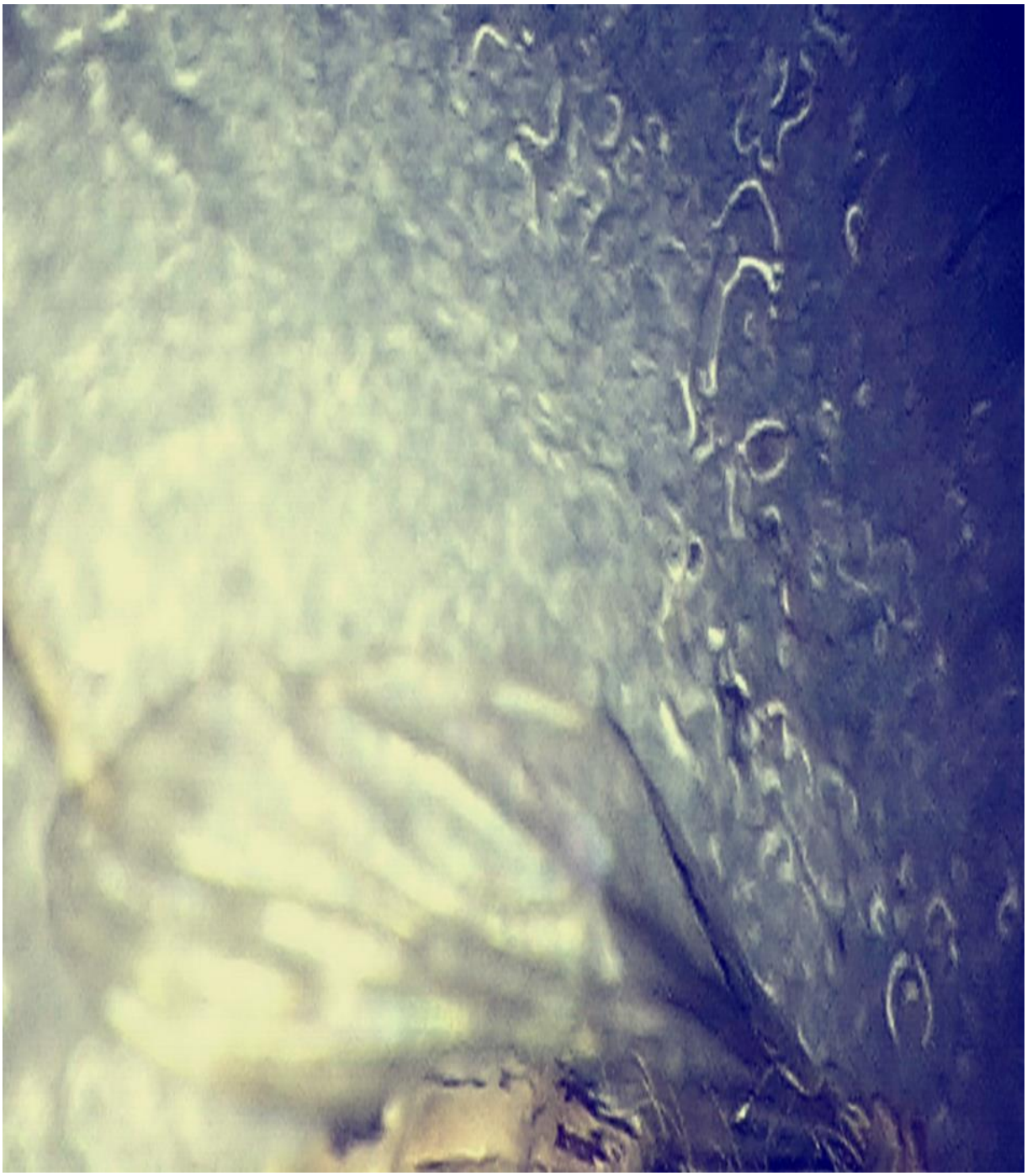

Plate V: Wing 2 (with different photographic effect) showing black veins

Plate VI: Photographic effect showing brown or blackish scales on the mosquito's abdomen

Appendix II: Protocol for DNA Extraction

1. For grinding specimens, use a razor blade to cut off the cap and rim of a 0.67 mL micro centrifuge tube. Make a pestle by inserting a 1.5 mL blue pipette tip into the smaller 0.67 mL micro centrifuge tube

2. Rinse alcohol-preserved specimens in sterile distilled water briefly to remove excess alcohol.

3. Grind animal on inside wall of larger tube (with pestle) in 20-30 µL of Lysis buffer. Once sufficiently ground, add Lysis buffer to a total volume of 1 mL. Add 100 µL of 10% SDS and 4 µL of Proteinase K (20 mg/mL) [keep enzyme mixture on ice] and mix.

4. Incubate at 55°C with rotation/shaking for a minimum of 4 hours to overnight.

5. Add 500 µL of equilibrated phenol (pH 8.0) and shake tube well for 5-10 minutes.

6. Centrifuge for 10 min at 12,000 rpm in a micro centrifuge.

7. Remove aqueous phase (usually the top layer) and place into fresh tube.

   Try not to collect any portion of the bottom layer when sample is transferred.

   Repeat steps 3-5 on the aqueous sample once (or until interface between lower organic phase and upper aqueous phase is clean).

8. Add 500 µL of chloroform, place on rotator for 5-10 min, and centrifuge for 10 min. Remove aqueous phase (top layer) and place in a new tube.

9. At this point you can stop and continue with the DNA precipitation steps at a later time, or amplify directly from the sample (if concerned about losing DNA at precipitation steps, storage is not recommended for a long period).

10. Add cold 100% ethanol to sample tube from step 6 above (fill to top).

11. Place at -80°C for 15 min.

12. Centrifuge for 25 min at 12,000 rpm in a micro centrifuge.

13. Discard alcohol carefully by pipetting without disturbing pellet (if pellet is visible). Check pipette tip before ejecting to ensure that pellet was not drawn into pipette tip during alcohol removal. If pellet is inside pipette tip, return pellet to tube and spin again.

14. Add 1000 µL of 70% ethanol and vortex for a few sec.

15. Centrifuge at 12,000 rpm in a micro centrifuge for 10 min. Check for pellet as above.

16. Air dry at room temperature or place in a vacuum centrifuge without heat.

17. Re-suspend DNA pellet in 100 µL sterile water.

18. Place DNA sample in a 50-55°C water bath for 5-10 min to dissolve DNA pellet before amplification. Thoroughly mix DNA sample before any further procedures. Use 1-2 µL of sample for PCR.

19. Keep a working DNA sample in the refrigerator (i.e., 30 µL aliquot) to avoid freeze/thaw cycles and place remainder of DNA sample at -20°C for use.

Appendix III: **Final alignment of 15 nucleotide sequences**

>5A_16S

TATTAATTAATTATTATGTTATAGTACGAAAGGACCTCAAAAAAGCCCTATAGATCTT

AATAATAGATAAATATAAATTTTAAAGAATTTTAAATTGATAGATTAATTAAAAAATTT

TATAGGGGTGAAATATAATTTTAAAATTCTTTTATTTTTTGTTTTATCTAGATTTTATG

AAAAAAATAATTCCAAGTTTATTGGTTGTAATTTTTGTTATACTATGAGTTG

>9A_16S

TATTAATTAATTATTATGTTATAGTACGAAAGGACCTCAAAAAAGCCCTATAGATCTT

AATAATAGATAAATATAAATTTTAAAGAATTTTAAATTGATAGATTAATTAAAAAATTT

TATAGGGGTGAAATATAATTTTAAAATTCTTTTATTTTTTGTTTTATCTAGATTTTATG

AAAAAAATAATTCCAAGTTTATTGGTTGTAATTTTTGTTATACTATGAGTTG

>12A_16S

TATTAATTAATTATTATGTTATAGTACGAAAGGACCTCAAAAAAGCCCTATAGATCTT

AATAATAGATAAATATAAATTTTAAAGAATTTTAAATTGATAGATTAATTAAAAAATTT

TATAGGGGTGAAATATAATTTTAAAATTCTTTTATTTTTTGTTTTATCTAGATTTTATG

AAAAAAATAATTCCAAGTTTATTGGTTGTAATTTTTGTTATACTATGAGTTG

>MF172536_Anopheles_eiseni

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>DQ202298_Anopheles_maculatus

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>MF172525_Aedes_aegypti

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>MF172526_Aedes_aegypti

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AAATATTTGATCCAGTTATATTGGTTGAAATTTTAAGTTACCTTAGGGATA

>MF172528_Aedes_arborealis

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ATTGGGGTGATATATAATTTTAAATAACTTTTATTTTTTTTTTTTACATTAATATATGAA

AATATTTGATCCAATAATATTGGTTGAAATTTTAAGTTACCTTAGGGATA

>MF172545_Culex_coronator

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>EU283340_Culex_pipiens_quinquefasciatus

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TATTGGGGTGATATATAATTTTAAAAAACTTTTAAATTTTTATTTAACATAAATATATG

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>EU339145_Culex_quinquefasciatus

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>EU339147_Culex_quinquefasciatus

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>MF172625_Sabethes_undosus

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>MF172624_Sabethes_undosus

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ATGAATATTATTGATCCAATTTTATTGATTAAAAAATTAAGTTACCTTAGGGAT

Appendix IV**: Questionnaires (English and Setswana versions)**

QUESTIONNAIRE/ INTERVIEW GUIDE (English version)

Malaria in Nata village in the northern Botswana: Evaluation of vector control methods

Dear Respondent

My name is Oduetse Ivan Nkaiwa, I am a student at Central University of Technology-Free State, SA. I am doing my Masters in Environmental Health and working on a research study on Malaria disease in Nata village.

As part of the study, this questionnaire is to get as much information as possible from you about the knowledge and your views towards the malaria control methods used in your village. The duration of the questionnaire will be approximately 15 minutes. The results of the study will be used to identify those negative factors brought by these methods and also bring and identify factors that can be done to improve all the negative factors.

Please be assured that all the information discussed will be treated with utmost confidentiality and will be used on improving strategies of reducing the burden of malaria in your community. Please be further informed that where you will feel uncomfortable to answer any question you are welcome to do so.

Thank you for your time.

The questionnaire contains section A and B

Date interview conducted: _____ Place of Interview: _____

Instruction: please indicate your response by tick

SECTION A: DEMOGRAPHIC DATA

Gender: Male _____ Female _____

Age: _____

SECTION B: KNOWLEDGE OF MALARIA CONTROL METHODS

Please indicate your response by a tick

1. Do you know any malaria control methods used in your village? Yes No

Please name them if YES

_____

_____

_____

_____

_____

_____

2. Which one (name above) do you prefer and WHY?

_____

_____

_____

_____

_____

3. What are your views on the methods of malaria control you named above?

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

4. Do you think the methods contaminate the environment or food? Yes No

Please explain if Yes

_____

_____

_____

_____

_____

Thank you for time.

AUTHORIZATION

_____ DATE: _____

Participant's signature

_____ DATE: _____

Researcher's signature

QUESTIONNAIRE/ INTERVIEW GUIDE (Setswana version)

Bolwetsi ja letshoroma mo motseng wa Nata mo bokone jwa Botswana: Tekanyetso ya mefuta ya go lwantshwa bolwetsi.

Go Motsayakarolo

Leina lame ke Oduetse Ivan Nkaiwa, moithuti kwa Central University of Technology-Free State. Ke dira dithuto tsa Masters in Environmental Health gape ke dira dipatlisiso ka bolwetse ja letshoroma mo motseng wa Nata.

E le bontlha bongwe ja dipatlisiso tse,potsolotso e ke go leka ka bojotlhe go kopa kana go tsaya dikitso mo go wena ka mefuta ya go lwantsha bolwetse jo e dirisiwang mo motseng wa gago. Potsoloso kana puisano e tla tsaya metsotso e le lesome le botlhano. Maduo a dipatlisiso tse a tla dirisiwa go tokafatsa tse di sa siamamng kana dikgoreletsi tse di bakiwang ke mefuta e le go tlisa le go bona tse di ka dirwang go tokafatsa tse di dikgwetlho.

Ke go tlhomamisetsa gore tse re tla di buang fa , e tla nna e le sephiri magareng ga rona and e tla nna kitso e tla dirisiwang go tokafatsa ntwa ya go lwantsha bolwetse ja letshoroma mo motseng wa lona.

Ke lebogela nako ya gago

Puisanyo ya rona e nale dikarolo tse pedi

Letsatsi: _____ Lefelo : _____

Kaelo: Supa ka go tshwaya

KAROLO A: KA GA MOTSAYA KAROLO

Bong: Rre _____ Mme _____

Dingwaga: _____

KAROLO B: KITSO KA MEFUTA YA GO LWANTSHA BOLWETSE JA LETSOROMA

Ka tswee tswee supa karabo ka go tshwaya

1. A o itse mefuta ya go lwantsha bolwetse ja letshoroma e dirwang mo motseng wa gago? Ee Nnyaa

Fa karabo e le Ee, di nankole

_____

_____

_____

_____

_____

_____

_____

_____

2. Mo mefuteng ya go lwantswa bolwe ja letsoroma e o e nankotseng,ke ofe mofuta o o go siametseng? Tlhalosa gape gore ka goreng?

_____

_____

_____

_____

_____

_____

3. Mo mefuteng e o e nankotseng fa godimo,maikutlo a gago ke afe mo mefuteng e dirisiwang go lwantsha bolwetse ja letshoroma

_____

_____

_____

_____

_____

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_____

_____

_____

_____

_____

4. A o akanya gore mefuta e dirisiwang go lwantsha blwetse ja letshoroma e ama kana e kgotlela tikologo kana dijo? Ee Nnya

Fa karabo e le Ee, tlhalosa

_____

_____

_____

_____

Ke lebogela go bo o tsere nako ya gago go bua le nna

TETLELELO

_____ Letsatsi: _____

Monwana wa Motsayakarolo

_____ Letsatsi: _____

Monwana wa Mmatlisisi