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Identification of arboviruses circulating in mosquito populations in the Bloemfontein area, South Africa

Gert Ignatius du Preez Terblanche

Adult Aedes albopictus (Armstrong, 2012)

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Identification of arboviruses circulating in mosquito populations in the Bloemfontein area, South Africa

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B.Sc. (Honours)

Submitted in fulfilment of the requirements for the degree MMedSc Virology completed in the Division of Virology, in the Faculty of Health Sciences, University of the Free State

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University of the Free State, Bloemfontein, South Africa

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Declarations

I, Gert Ignatius du Preez Terblanche declare that the master's research dissertation that I herewith submit at the University of the Free State, is my independent work and that I have not previously submitted it for a qualification at another institution of higher education.

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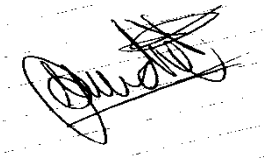
Signature: 

Abstract

Globally there are more than 3 500 different species of mosquito. Many of these are known to be the primary insect vectors of many medically important diseases. Adequate surveillance programs should be put in place to develop effective control strategies and to prevent outbreaks of disease. For a surveillance programme to be effective, mosquito vectors need to be identified accurately. This is done through combining morphological, molecular and environmental data to get more accurate identification results. Currently the diversity of mosquito populations circulating in the Bloemfontein area is not well defined.

Mosquitoes were captured from three different sites in the Bloemfontein area. A total of 318 mosquitoes were collected in four different genera. A total of ten different species were identified using morphological identification. Six specimens could only be identified to genus level, because of extensive damage to their external anatomy. Representative specimens were selected from selected species. These included *Anopheles squamosus, Culex theileri, Aedes aegypti, Mansonia uniformis* and two *Aedes* subgenus *Ochlerotatus* species. The *Ochlerotatus* species include *Ochlerotatus harrisoni* and *Ochlerotatus juppi*. DNA was extracted from these mosquitoes and sequenced bidirectionally making use of the barcoding primers, HCO2198 and LCO1490. *Anopheles squamosus* and *Aedes aegypti* were identified successfully using the barcoding primers. The primers were less useful for obtaining adequate sequence data for genetic identification of *Ochlerotatus* spp., *Culex theileri* and *Mansonia uniformis* and it is proposed that additional sequence data be obtained subsequent to cloning of fragments.

The field caught mosquitoes were sorted and pooled, according to species, capture site and capture date. An RT-qPCR assay was developed to detect Sindbis virus (SINV) using a primer and probe set specifically targeting a region of the nsp2 gene. Another RT-qPCR assay was developed to detect West Nile virus (WNV) and Wesselsbron virus (WSLV) using a primer and probe set targeting a region of the NS5 gene. The assays were validated using cDNA reverse transcribed from RNA extracted from wild caught mosquitoes to ensure that there were no inhibitors in the mosquitoes that would interfere with downstream reactions. These assays proved to work

efficiently. RNA controls were constructed for WNV and WSLV to be used as the positive controls to validate the RT-qPCR assays.

RNA was extracted from mosquito pools and was screened using the RT-qPCR assays. All mosquito pools tested negative for the arboviruses that were screened for. Due to the small sample size and a low infectivity rate of mosquitoes, it is not surprising that no viral RNA was detected in this study. The number of mosquitoes caught in this study is too low to be used for surveillance but was rather used as a proof of concept for developing appropriate assays. Large scale surveillance programmes will be needed to determine the full extent of arbovirus circulation in the Bloemfontein area, Free State province, South Africa and the assays developed in this study can be used to execute such programmes.

Key words: DNA Barcoding, mosquito, morphology, *Anopheles squamosus*, alphaviruses, flaviviruses, West Nile virus, Sindbis virus, Wesselsbron virus, RT-qPCR,

Presentations

2016: Presentation at the Institutional three-minute thesis competition for the master's category, University of the Free State.

2018: Presentation at the FameLab Free State regional heats, presenting amongst the top ten students in the Provincial category. Central University of Technology.

2018: Presentation at the Department of Health, Free State Health day. University of the Free State.

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List of Abbreviations

°C – degrees Celsius

6-Fam – 6-carboxyfluorescein

A – adenine

amp -ampicillin

ATP – adenosine triphosphate

BFV – Barmah Forest virus

BLAST – Basic Local Alignment Search Tool

BOLD – Barcode of Life Database

bp – base pairs

BR – broad range

C – capsid

C – carbon

C – cytosine

CDC – Center for Disease Control

cDNA – complementary deoxyribonucleic acid

CHIKV – chikungunya virus

COI – cytochrome oxidase sub-unit 1

CTP – cytidine triphosphate

DENV – dengue fever virus

DNA – deoxyribonucleic acid

DNase 1 – deoxyribonuclease 1

ds – double stranded

DTT – dithiothreitol

E – east

E protein – envelope protein

EDTA – ethylene diamine tetra-acetic acid

EEEV – eastern equine encephalitis virus

ER – endoplasmic reticulum

FSNBG – Free State National Botanical Gardens

g – gram

G – guanine

GTP – guanosine triphosphate

IABkFQ – IOWA Black™ FQ quencher

IPTG – isopropyl β–D–thionalactopyronoside

ITS2 – inter-spacer region 2

JEV - Japanese encephalitis virus

kb – kilobase

kDa – kilo Dalton

km – kilometer

LB – Luria Bertani

LNA – locked nucleic acid

MAYV – Mayaro virus

ml – milliliter

mM – millimolar

$MnCl_2$ – Manganese chloride

mRNA – messenger ribonucleic acid

MVEV – Murray Valley encephalitis virus

N – nitrogen

NADH – nicotinamide adenine dinucleotide dehydrogenase

NCR – non-coding region

nfw – nuclease free water

NICD – National Institute for Communicable Diseases

nm – nanometer

nsp – non-structural protein

O – oxygen

ONNV – o'nyong nyong virus

ORF – open reading frame

PCR – polymerase chain reaction

pmol – picomole

poly A – polyadenylation A

prM – pre-cursor membrane

RNA – ribonucleic acid

rpm – revolutions per minute

RRV – Ross River virus

RVF – Rift Valley fever

RVFV – Rift Valley fever virus

S – South

SFV – Semliki Forest virus

SINV – Sindbis virus

SLEV – St. Louis encephalitis virus

SOC – super optimal broth with catabolite repression

T – thymidine

TAE – Tris acetate EDTA

Taq – *Thermus aquaticus* DNA polymerase

Tm – melting temperature

TTP – thymidine triphosphate

U – units

UV – ultra-violet

V – volts

VBDF – vector-borne disease fieldwork

VEEV- Venezualan equine encephalitis virus

WEEV – western equine encephalitis virus

WHO – World Health Organization

WNV – West Nile virus

WSLV – Wesselsbron virus

x g - gravitational force

X-gal – 5-bromo–4–chloro–3-indoyl–β–D-galactopyronoside

YFV – yellow fever virus

ZIKV – Zika virus

µg – micrograms

µg – micrograms

µl – microliter

µM – micromolar

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Chapter 1 – Literature Review, problem statement, aim and objectives

1.1 Literature review

1.1.1 Introduction

An arthropod- borne virus (arbovirus) is any virus that is transmitted from one vertebrate host to another via an arthropod. These viruses are typically maintained in natural cycles between haematophagous insects and their vertebrate hosts (Calisher, 1994). This includes insects belonging to the order Diptera, such as mosquitoes, sand-flies, black flies and biting midges (Franz *et al*., 2015). Of these insects, mosquitoes are the most medically significant group (Beerntsen *et al.*, 2000, Burt *et al*., 2014; Franz *et al*., 2015). Non-insect arthropods, such as ticks, are also involved in the transmission of arboviruses. Arboviruses are a large group of diverse viruses belonging to different viral families (Arpino *et al.*, 2009), with different life cycles and modes of transmission. Many arboviruses are known to cause acute disease in humans and in recent years have been responsible for large outbreaks of human disease. These viruses are transmitted to vertebrate hosts during blood feeding (Calisher, 1994). If the vertebrate host is susceptible to infection, the virus will multiply rapidly. When viraemia is above a certain threshold level, the virus can be transmitted to other blood feeding vectors (Mattingly, 1969). Some hosts act as the main source of infection for vectors. These hosts act as reservoirs for virus and can be responsible for the amplification and spread of viruses. Other hosts do not act as a source of infection for vectors, but they can develop disease. Humans and many domestic animals are frequently not the main source of infection for vectors but can develop symptoms of disease. Infection in humans is usually incidental and does not play a significant role in the main cycles of disease (Calisher, 1994).

A primary requirement for vector competence is the ability of the virus to replicate in the mid-gut wall of the arthropod (Mattingly, 1969). In mosquitoes, only females take

a blood meal. The blood meals are needed to fulfil the protein needs of the females during egg production. This explains why arboviruses are mostly isolated from female mosquitoes (Calisher, 1994). Arboviruses can be maintained in vectors in a few different ways. The main cycle is a normal vector-host-vector transmission. In a few instances' virus, can be maintained without the presence of a host (Mattingly, 1969; Calisher, 1994). This can be either trans-stadial or trans-ovarial transmission (Calisher, 1994).

Most arboviruses that are responsible for severe morbidity and mortality in humans worldwide are members of the *Flaviviridae*, *Togaviridae* or *Bunyaviridae* viral families (Franz *et al*., 2015).

1.1.2 Mosquito classification and taxonomy

Globally there are more than 3 500 mosquito species, with the majority found in tropical and sub-tropical regions of the world (Reiter, 2001; Service, 2004; Harbach, 2008; Rozo-Lopez & Mengual, 2015; Beebe, 2018). Mosquitoes belong to the order Diptera or the two-winged flies (Clements,1992; Harbach, 2008; Lawrence, 2011; Beebe, 2018). All of them are placed into a single family, Culicidae. The family represents a monophyletic taxon (Harbach, 2007), that can be further divided into three sub-families and a total of 113 different genera (Harbach, 2008). These three sub-families include: Toxorhynchitinae, Anophelinae and Culicinae (Service, 2004). Mosquitoes are one of the most primitive families within the Diptera order, grouping more closely to gnats, midges and crane flies, than to real flies (Clements, 1992). Mosquitoes can be found globally, except in areas that are permanently frozen (Reiter, 2001).

Mosquitoes can be easily distinguished from other flies based on a few characters: they have a conspicuous forward projecting proboscis, they possess numerous appressed scales on their thorax, legs, abdomen and wing veins and finally they also have a fringe of scales along the posterior margin of the wings (Service, 2004).

1.1.3. Mosquito morphology

The morphology of mosquitoes is very important, because morphological characters are used to identify mature larvae and adults (Harbach, 2007).

Mosquitoes are relatively small insects with a slender body, long legs and the presence of scales on the body surface (Rozendaal, 1997; Service, 2004; Harbach, 2007). These insects usually measure between 3-6 mm in length, but sizes do range from 2-19 mm. Like all other insects the body is divided into a head, thorax and abdomen. In mosquitoes these body structures are quite distinctive (Rozendaal, 1997; Service, 2004).

On the head is a pair of distinctive kidney shaped compound eyes. A pair of segmented, filamentous antenna is situated between these eyes. These antennae differ between the two sexes. Female mosquitos have short, pilose antennae, while male mosquitoes have plumose antennae. Plumose antennae have various long hairs. Directly beneath the antennae is a pair of palps. These palps differ in length as well as structure depending on the sex and species of the mosquito (Service, 2004).

Below the palps are the mouthparts. In mosquitoes the mouthparts are in the form of an elongated proboscis. This is one of the most characteristic features of mosquitoes (Harbach, 2007). The proboscis in mosquitoes' projects forward. This is the case for both sexes, even though males do not bite (Service, 2004).

The largest part of the mouthparts is the long and flexible gutter-shaped labium. The labium terminates in the labella, which is a pair of small flap-like structures. The labium nearly encircles the other mouthparts, which plays an important role in protecting the other mouthparts against damage. The top structure of the mouthparts is known as the labrum. It is slender and pointed and it has a groove on its ventral surface. Between the labrum and the labium are a few needle-like structures, including: a lower pair of toothed maxillae, an upper pair of untoothed mandibles and a single, untoothed, hollow stylet known as the hypopharynx (Service, 2004).

When a female mosquito bites a host, the labella are placed on the skin surface. The labium does not penetrate the skin but folds back and exposes the internal mouthpart

structures and allows the maxillae, mandibles, hypopharynx and labrum to penetrate the skin (Service, 2004).

Saliva of the mosquito is produced in a pair of trilobed salivary glands that are situated on the ventral side in the front part of the thorax. The saliva is transported through the hypopharynx. The saliva contains anti-haemostatic enzymes that facilitate the uptake of blood. Anti-coagulants prevent the blood from clotting and obstructing the mouthparts. Anaesthetic substances ensure that the bite is not painful and, therefore, lowers the host's defense reactions. In males the maxillae and mandibles are reduced, therefore they cannot bite (Service, 2004).

The thorax of mosquitoes is covered in scales on the dorsal and marginal surfaces. These scales can be shiny or dull and can be nearly any colour. The arrangement of these different coloured scales gives some species distinctive patterns (Service, 2004).

In mosquitoes, only the pair of fore wings act as functional wings. These wings are a pair of membranous wings that are used by the mosquito for flying. These wings are long and relatively narrow. The number and arrangement of wing veins in mosquitoes is relatively uniform. These veins are covered in scales that are usually black, brown, white or creamy white in colour. The shape of these scales and the way they are arranged in is different between different genera and species of mosquito. On the posterior edge of the wing is a fringe of scales. When a mosquito is at rest these pair of wings fold back onto themselves in a scissor blade orientation (Service, 2004). The pair of hind wings is reduced into small structures better known as halteres. These halteres act as balancing organs (Service, 2004).

The legs of mosquitoes originate on the thorax of the mosquito. These legs are long and slender, and they are covered in scales. The scales can be different colours and they are normally arranged in patterns, usually in the form of rings. The tarsi of the mosquitoes usually end in a pair of claws. These claws can be smooth or toothed depending on the species of mosquito. In some species a pair of small fleshy pulvilli can be found between the claws. The pulvilli allow the mosquito to sit on nearly any surface (Service, 2004).

The abdomen is divided into 10 segments. Only the first seven or eight segments are visible. In females the last abdominal segment terminates in a pair of cerci, whilst in

males' prominent claspers are present that form part of the external genitalia (Service, 2004).

The larvae of mosquitoes can easily be distinguished from the larvae of other aquatic insects (Harbach, 2007). They do not possess legs, but do possess a distinct head and antennae, a bulbous thorax, posterior anal papillae and either a pair of respiratory openings (subfamily Anophelinae) or an elongate siphon (subfamily Culicinae). This siphon is located on the end of the abdomen (Harbach, 2007).

1.1.4. Mosquito biology

Mosquitoes are found in all types of habitats, including terrestrial and aquatic environments. They have many behavioural and morphological adaptations to adapt to these different habitats (Rozo-Lopez & Mengual, 2015). Due to their delicate nature, mosquitoes will mostly be found in areas where the air is relatively cool, and humidity is high. Many mosquito species are found just a few meters off the ground, whilst sylvatic species are found nearly exclusively in the forest canopy (Harbach, 2007). Mosquitoes are, however, also able to survive elevations of up to 1250 m above sea level (Service 2004). The vertical distribution of mosquitoes is dictated by their feeding preferences, being found in the areas where their hosts spend most of their time (Harbach, 2007).

Different mosquito species have different times of flight and feeding activity. These behavioural aspects are quite species-specific. Mosquitoes can therefore be nocturnal, crepuscular or diurnal (Rozendaal, 1997; Harbach, 2007). Some mosquito species are very selective in their host range feeding on a single host species or on a few closely related host species. Other mosquitoes feed on a wide variety of hosts alternating between reptiles, birds and mammals (Reiter, 2001). Mosquitoes also differ in their association with humans. Certain species mostly bite indoors and others mostly outdoors (Rozendaal, 1997).

Like all other true flies, mosquitoes have a holometabolic life cycle, which means that the juvenile forms are totally different to the adult insect. The larvae and pupae are aquatic while the adults are free-flying, they have different habitat preferences and they will utilize different food sources. Transformation into the functional adult form

takes place during pupal development which is the non-feeding stage (Clements, 1992). Mosquitoes have four distinct stages in their life cycle, egg, larvae, pupae and adults (Rozendaal, 1997).

The spectrum of larval habitats varies quite extensively (Clements, 1992; Service, 2004; Harbach, 2007). Mosquitoes can primarily be found in temporary or permanent bodies of ground water (Harbach, 2007). Some prefer large and usually permanent collections of water such as swamps, marshes, rice fields and burrow pits. Others prefer smaller collections of water such as pools, puddles, drains, or gutters (Clements, 1992; Service, 2004).

Various natural water containers also act as breeding sites for different mosquito species: tree holes, water-filled bamboo stumps, rock pools, fallen leaves, flower brachts, bromeliads, coconut husks, water filled snail shells, pitcher plants and leaf axils in bananas, pineapples and other plants (Clements, 1992; Service, 2004; Harbach, 2007). Mosquito larvae normally choose to live in stagnant water bodies or water bodies where water movements are at a minimum (Clements, 1992).

Artificial water containers are also suitable habitats, like wells, clay water pots, tin cans and car tires (Service, 2004; Harbach, 2007). Certain larvae prefer shaded areas, whilst others prefer sun-lit areas. Many mosquito larvae cannot survive in polluted water, whilst some breed in water containing excreta and rotting plant material (Service, 2004). Certain mosquito species choose to breed in brackish or salt water like salt water marshes, most mosquitoes do however choose to live in fresh water habitats (Clements, 1992).

1.1.5. Vector competence

Vector competence refers to the intrinsic permissiveness of an arthropod vector to infection, replication and transmission of a virus (Bosio *et al.*, 2000, Bennett *et al.*, 2002, Goddard *et al.*, 2002). Population density, host preference, longevity, temporal and spatial behaviour, feeding time and behaviour and seasonal activity of mosquitoes play an important role in determining whether a mosquito is a vector for a specific virus (Sardelis *et al.*, 2001).

After being taken up by a mosquito during a blood meal, a virus encounters several different barriers that it will need to overcome to be able to multiply (Bosio *et al.*, 2000). Blood feeding arthropods have developed several different mechanisms to ensure that blood from the host animal flows without any difficulty. Mosquitoes feed very effectively, because they have anti-haemostatic mechanisms in their saliva. Different mosquito species exhibit these mechanisms to differing levels. In many mosquitoes, the coagulation of blood in the midgut may act as a barrier to infection. Blood can become too thick for the pathogen to migrate to the midgut wall, where replication will take place (Beerntsen *et al.*, 2000).

After ingestion, pathogens can face several different pharyngeal and cibarial armature as they travel through the mosquito digestive system. For larger parasites, these structures can cause physical damage. This will influence the infection rates and severity. This is important in mosquitoes when we consider larger parasites and microfilariae (Beerntsen *et al.*, 2000). Upon entry, a virus needs to establish infection in the midgut of its mosquito vector (Bosio *et al.*, 2000). In the midgut, many proteolytic enzymes are excreted into the lumen. These enzymes can have an impact on the pathogens and in turn influence vector competence (Beerntsen *et al.*, 2000). In the midgut, a peritrophic membrane is formed. This membrane encapsulates the blood meal and therefore separates any pathogens in the blood meal from the midgut epithelium (Beerntsen *et al.*, 2000). After the virus has completed replication in the midgut epithelium, it needs to pass through the midgut escape barrier to complete replication in other body tissues. Viruses need to pass through the haemolymph-filled haemocoel. The haemolymph acts as the primary site of immunity, where any foreign material is recognised. As soon as non-self-material is recognized defence responses are initiated. In the haemolymph haemocytes play the roles of recognition, encapsulation and phagocytosis. These cells can also produce enzymes that kill parasites (Beerntsen *et al.*, 2000).

After replication in the gut, viruses normally spread to other, surrounding tissues. Most viruses seem to have an affinity for nervous tissue or the salivary glands. Moving to these tissues makes it possible for viruses to be transmitted trans-stadially. In mosquitoes this may not be of major importance in disease transmission (Mattingly,

1969). For biological transmission to vertebrate hosts, the virus needs to infect the salivary glands, which requires evasion of a salivary gland infection barrier (Franz *et al.,* 2015). Viral particles will then be shed to the next host during the next blood meal (Bosio *et al.*, 2000).

1.1.6. Mosquitoes of the Free State Province, South Africa

All the species in the genus *Aedes* that have been previously found in the Free State province are listed in Table 1.1.

Table 1.1: Species list of mosquitoes in the genus *Aedes* that have previously been collected in the Free State province, South Africa.

Species Name	Reference
Aedes dentatus	Muspratt 1953
Aedes durbanensis	Jupp *et al.* 1980
Aedes mixtus	Muspratt 1953
Aedes hirsutus	Muspratt 1953
Aedes natronius	van der Linde *et al.* 1982
Aedes vittatus	van Staden 1992
Aedes sudanensis	van der Linde *et al.* 1982
Aedes circumluteolus	Edwards 1941
Aedes luridus	McIntosh 1971
Aedes luteolateralis	van der Linde *et al.* 1982
Aedes mcintoshi	Huang 1985
Aedes unidentatus	McIntosh 1971
Aedes caballus	Jupp *et al.* 1980; McIntosh 1973
Aedes juppi	Jupp *et al.* 1980; McIntosh 1973
Aedes aegypti	Jupp *et al.* 1980

Several species in the genus *Culex* have been collected from the Free State province. These species are listed in Table 1.2.

Table 1.2: Species list of mosquitoes in the genus *Culex* previously collected in the Free State province, South Africa.

Species Name	Reference
Culex lineata	van der Linde *et al.* 1982
Culex annulioris	Muspratt 1953
Culex pipiens	Jupp 1978; Jupp *et al.* 1980
Culex poicilipes	van der Linde *et al.* 1982
Culex quinquefasciatus	Jupp 1978; Muspratt 1953
Culex theileri	Muspratt 1953
Culex univittatus	Muspratt 1953
Culex tigripes	Jupp 1978; Jupp *et al.* 1980
Culex salisburiensis	Jupp *et al.* 1980
Culex salisburiensis ssp naudeanus	Jupp *et al.* 1980

Only a single species in the genus *Anopheles* has been collected from the Free State province, South Africa – *Anopheles squamosus* (Jupp & Kemp, 1998).

Certain other species have also been isolated from the Free State province South Africa. A list of the other species can be seen in Table 1.3.

Table 1.3 Species list of mosquitoes in other genera previously collected in the Free State province, South Africa.

Species Name	Reference
Culiseta longiareolata	Jupp 1978; Muspratt 1953
Mansonia uniformis	van der Linde *et al.* 1982

1.1.7. The use of barcoding in the genetic identification of mosquitoes

Morphological identification is seen as the gold standard method for identification of mosquitoes (Erlank *et al.* 2018), but DNA barcoding can be used as a very efficient technique to supplement morphological identification. This method can be used to overcome some of the limitations involved with morphological identification (Batovska *et al.,* 2016). The use of genetic barcoding has provided a method to genetically identify mosquitoes based on the concept that each species has a unique genetic identity (Chan *et al.,* 2014). A DNA barcode is a short, standardised sequence of DNA that can be used as a genetic marker, for use in species identification (Che *et al.,*

2012; Chan *et al.,* 2014). These sequences have less intra-specific variance than inter-specific variance (Batovska *et al.,* 2016).

The main reason for the use of barcoding sequences is to achieve accurate and reliable identification of species, which is also true for morphological identification (Che *et al.,* 2012; Chan *et al.,* 2014). Barcoding of mosquitoes is advantageous, because it is a technique that can be easily standardised. This technique can also use very small pieces of tissue from any developmental stage of mosquito, from egg to adult (Cywinska *et al.,* 2006; Che *et al.,* 2012; Chan *et al.,* 2014). This technique can also be used to identify mosquitoes that are damaged or degraded. In certain instances voucher specimens will, however, need to be stored intact and DNA analysis will not be done on these specimens. For the most accurate identification of mosquitoes, morphological, molecular and ecological data should be combined (Chan *et al.,* 2014; Erlank *et al.*, 2018).

1.1.8. Alphavirus introduction and brief history

Alphaviruses are small relatively simple, enveloped, positive-sense RNA viruses (Griffin, 1998, Weaver *et al.,* 2012). Alphaviruses have been implicated as the aetiological agent of both human and animal disease. These viruses commonly cause symptoms including fever, rash and arthritis in their hosts (Jose *et al.*, 2009). Some of the most common members in this group include the equine encephalitis viruses in humans and horses in the Americas, Ross River virus (RRV) in Australia and chikungunya virus (CHIKV) in Africa, Asia and more recently in South America (Strauss *et al*.,1995).

1.1.9 Alphavirus classification and molecular characteristics

The family *Togaviridae* can be sub-divided into two separate genera: the *Alphavirus* and *Rubivirus* genera. The *Rubivirus* genus is comprised of a single species, *Rubella virus* (Jose *et* al., 2009; Weaver *et al.*, 2012). The *Alphavirus* genus contains 29 recognized species (Forrester *et al.*, 2012; Weaver *et al.,* 2012). This genus can be

divided into two main groups, which includes the Old-World and New World alphaviruses. This classification is broadly defined according to the geographical distribution of these viruses and the presentation of disease caused (Jose *et al.*, 2009; Leung *et al.,* 2011). Old-World alphavirus infections in mammals are mostly involved in diseases that present with fever, rash, and arthritic symptoms, mostly with high-titer viraemia and rarely fatalities, whereas New World alphaviruses are associated more with encephalitis and neurological diseases in horses and humans (Jose *et al.*, 2009; Leung *et al.,* 2011; van Niekerk *et al.,* 2015).

The alphaviruses are related to each other in terms of genome organization, structure of the virion, sequence similarity and serology (Rayner *et al.,* 2002). It has been proposed that several trans-oceanic exchanges have occurred in the past and that it was most likely mediated by the movements of migratory birds (Jose *et* al., 2009).

The alphaviruses were initially grouped according to antigenic relationships. These relationships were determined through serological assays. The alphaviruses were divided into seven antigenic complexes of mosquito-borne viruses. These complexes included the Venezuelan equine encephalitis virus (VEEV), Western equine encephalitis virus (WEEV) and Eastern equine encephalitis virus (EEEV), Semliki forest virus (SFV), Barmah forest virus (BFV) and Middelburg virus (MIDV) (Weaver *et al.,* 2012).

Alphaviruses are small, spherical particles that are approximately 65-70 nm in diameter and have an icosahedral structure (Mukhopadhyay *et al.,* 2006; Jose *et* al., 2009; Forrester *et al.*, 2012; Weaver *et al*., 2012). These viruses have an envelope glycoprotein shell that is embedded in a plasma membrane derived envelope. The envelope is studded with membrane anchored glycoproteins (E1 and E2) (Ryman & Klimstra, 2008). The virion envelope contains a lipid bilayer that is derived from the plasma membrane of the host that it infects (Mukhopadhyay *et al.* 2006; Jose *et* al. 2009). The nucleocapsid of these viruses contain 240 capsid (C) proteins. The glycoproteins are arranged in 80 trimer spikes. These spikes are made up out of three E1/E2 heterodimers (Weaver *et al.,* 2012).

The genome is comprised of a single copy of single stranded, positive-sense RNA that is approximately 11.5 kb long (Mukhopadhyay *et al.,* 2006; Powers & Logue, 2007;

Ryman & Klimstra, 2008; Jose *et* al., 2009; Forrester *et al.*, 2012; Vander Veen *et al.*, 2012; Weaver *et al.,* 2012).

The genome contains two open reading frames that encode four non-structural proteins and five structural proteins (Rayner, 2002; Forrester *et al.*, 2012, Weaver *et al.,* 2012).

The structural proteins include the capsid, E3, E2, 6K and E1 proteins (Mukhopadhyay *et al.,* 2006; Vander Veen *et al.,* 2012). The two reading frames are divided by the promotor region for the sub-genomic mRNA (Ryman & Klimstra, 2008). The genome organization of alphaviruses can be seen in Figure 1.1.

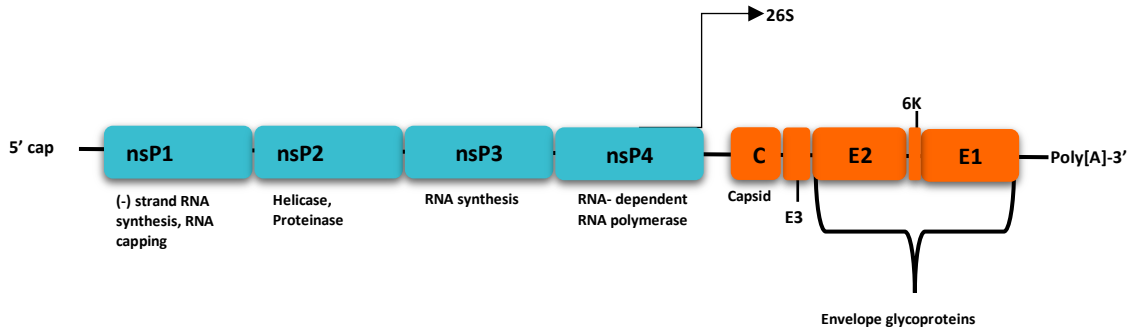

Figure 1.1: Genome organization of alphaviruses showing structural proteins (orange) and non-structural proteins (blue), with the functions of the proteins included.

The non-structural proteins are transcribed from the positive-sense genomic RNA and they function to transcribe full length negative-sense RNA. The negative sense RNA is a template for both additional genomic RNA as well as 26S sub-genomic mRNA (Vander Veen *et al.,* 2012). These proteins play a crucial role in virus replication. This in turn has an important influence on the pathogenesis of the virus (Mukhopadhyay *et al.,* 2006; Jose *et* al., 2009). The structural proteins are transcribed from the sub-genomic 26S mRNA as a polyprotein that is co-translationally and post-translationally cleaved to release the capsid protein and two mature envelope glycoproteins (E1 and E2) (Vander Veen *et al.,* 2012).

The genomic RNA will serve as the mRNA for the four nsP's (Shin *et al.,* 2012). Infection will commence when the four non-structural proteins are expressed as one of two polyproteins P123 or P1234. P1234 is expressed as a read-through of an opal terminator codon at the end of nsP3. Cleavage of these polyproteins is facilitated by a

protease in the nsP2. After translation of P1234 the polyprotein gets cleaved between P3 and 4 in a cis or trans orientation and P1 and 2 in the cis orientation. This will result in P123 + nsP4 and nsP1 + P23 + nsP4. These intermediates will synthesize negative strand viral RNA. Cleavage between P23 will switch on the synthesis of positive sense genomic and sub-genomic RNA's (Shin *et al.,* 2012). The shift to the synthesis of positive sense RNA happens within the first three hours. This newly formed RNA will continue to generate progeny genomes. From the structural polyprotein, the capsid protein is cleaved into the cytoplasm. The remaining polyprotein is processed and cleaved in the secretory pathway to yield the E1 and E2 glycoproteins that play an important role in the packaging of the virus (Ryman & Klimstra, 2008).

1.1.10. Epidemiology, prevalence and transmission of Alphaviruses

Most alphaviruses are transmitted biologically by haematophagous arthropods, especially mosquitoes, and replicate in both the arthropod vector and the vertebrate host (Jose *et* al., 2009; Weaver *et al.,* 2012). Most of the alphaviruses infect terrestrial animals, but there are exceptions to this. Salmon pancreatic disease virus and its sub-type infect farmed fish and cause an economic burden on the aquaculture industry. Southern elephant seal virus infects seals in Australia and has been isolated from lice. In general, these viruses are maintained in natural transmission cycles between susceptible vertebrate hosts, including rodents and birds, and arthropod vectors (Jose *et* al., 2009) in a classical arbovirus transmission cycle (Ryman & Klimstra, 2008; Weaver, *et al.,* 2012). In the arthropod vectors the viruses cause minimum physical effects and are a life-long persistent infection (Jose *et* al., 2009).

Mosquito-borne alphaviruses begin their infection in their human host with the deposition of the virus in the subcutaneous tissues. Replication occurs at the site of inoculation and the virus moves to the lymph nodes surrounding the inoculation site. Migration can occur in migratory cells such as Langerhans cells, dermal macrophages and dendritic cells, but it can also happen as free virus. The viruses amplify in the lymph nodes that drain the inoculation site. The primary viraemia in the serum is caused by the release of this virus into the efferent lymph. The efferent lymph system

drains into the circulatory system through the thoracic ducts to the subclavian vein and into the bloodstream. Using this pathway, it is possible for the virus to spread throughout the body infecting tissues far from the inoculation site. Development of a high viraemia in the peripheral blood system is essential for the reinfection of arthropod vectors (Ryman & Klimstra, 2008).

Diseases caused by alphaviruses have a typical epidemiology. Most of these diseases are linked to the biology of their reservoir hosts and mosquito vectors. Alphaviruses that have avian and rodent reservoir hosts are mostly found in constant enzootic cycles. The cycles mainly differ from typical cycles, because of weather conditions that affect the population dynamics of the mosquito vector. This can include changes in the availability of breeding sites and the availability of vertebrate animals to feed on (Weaver *et al*., 2012).

1.1.11. Clinical presentation, diagnosis and treatment of Alphaviruses

The Alphavirus genus is responsible for diseases including, febrile illness, encephalitis or polyarthritis ranging from mild to quite severe (Powers & Logue, 2007). This genus includes 29 species that are known to cause disease in humans (Powers & Logue, 2007). Some of the most medically significant alphaviruses, include the New World alphaviruses, VEEV, WEEV and EEEV, and the Old-World alphaviruses, Sindbis virus (SINV), SFV, RRV, chikungunya virus (CHIKV), BFV, o' nyong nyong virus (ONNV) and Mayaro virus (MAYV) (Ryman & Klimstra, 2008; Jose *et* al., 2009). Five alphaviruses have been isolated in southern Africa including: SINV, CHIKV, MIDV, SFV and Ndumu virus. CHIKV is the most widely distributed Old-World *Alphavirus* in the world, with SINV being the second most widely distributed (van Niekerk *et al.,* 2015).

The equine encephalitis viruses are found in the Americas, RRV is present in Australia and CHIKV was originally found in Africa but has subsequently spread to Asia and the Americas (Strauss *et al*., 1995). The viruses are responsible for millions of cases of serious disease. These diseases are primarily not life-threatening illnesses in humans (Ryman & Klimstra, 2008). The acute cases of Old-World viral infections are

characterized by fever, chills, headaches, eye pain, generalized myalgia, arthralgia, diarrhea, vomiting and rash. The viruses are closely related to the New World viruses, which may present with the same symptoms as the Old-World viruses in the acute stage of infection. New World viruses can, however, also cause encephalitis in humans and domestic animals (Ryman & Klimstra, 2008).

The initial diagnosis of alphavirus infection is generally reliant on the clinical presentation and medical history of patients (Sanchez-Seco *et al.* 2001). Alphavirus infections are diagnosed through the isolation or detection of virus in the serum or cerebrospinal fluid of patients during acute infection, from brain tissue in fatal encephalitis cases, the presence of IgM during the acute phase of infection or the change in total serum antibody levels of individuals between acute and convalescent phases. Viral detection methods include, the production of cytopathic effects on a variety of vertebrate cell cultures, the use of immunofluorescence and the detection of viral RNA using reverse transcription polymerase chain reaction (RT-PCR) (Weaver *et al.,* 2012).

1.1.12. Flavivirus introduction and brief history

The *Flavivirus* genus derives its name from the Latin word *Flavus,* which means yellow (Schlesinger & Schlesinger, 1986; Chambers *et al.,* 1990; Lindenbach *et al.,* 2007). This refers to the jaundice that is induced in patients that suffer from yellow fever, the first *Flavivirus* to be isolated (Schlesinger & Schlesinger, 1986; Chambers *et al.,* 1990; Lindenbach *et al.,* 2007). This prototype virus was described for the first time more than 100 years ago, when the scientist Walter Reed demonstrated that yellow fever can be experimentally transmitted to humans through the filtered serum of infected yellow fever patients. It was also found that the disease-causing agent is transmitted to humans through the bite of infected mosquitoes (Reed, 1902; Lindenbach *et al.,* 2007).

1.1.13. Flavivirus classification and molecular characteristics

The flaviviruses include approximately 70 known viruses of which most are arthropod-borne (Rice *et al.*, 1985, Hase *et al.,* 1989; Zanotto *et al.,* 1996). The *Flavivirus* genus is a large group of viruses that include some of the most medically significant human zoonotic viral pathogens (Schlesinger & Schlesinger, 1986; Kuhn *et al.,* 2002; Pettersson, 2013). These viruses are widely distributed globally (Schlesinger & Schlesinger, 1986; Pettersson, 2013). They are responsible for considerable morbidity and mortality and they may cause severe encephalitic, haemorrhagic, hepatic and febrile illnesses in a diverse range of vertebrates including humans (Zanotto *et al.,* 1996; Lindenbach *et al.,* 2007; Moureau *et al.,* 2007).

Understanding the evolution of viruses is very important in understanding the origin and spread of diseases (Gaunt *et al.,* 2001). Flaviviruses were initially classified as part of the *Togaviridae* family (Schlesinger & Schlesinger, 1986; Chambers *et al.,* 1990). In 1984 the International Committee for the Nomenclature of Viruses decided to make the *Flaviviridae* a family on its own (Schlesinger & Schlesinger, 1986). Positive-stranded RNA viruses within this family have been placed into three diverse super-families. This placement is based on the evolutionary relatedness of the RNA – dependent RNA polymerase (Lindenbach *et al.,* 2007). These viruses are further divided into antigenic complexes and sub-complexes. These complexes are constructed using serological cross reactivity. There are eight main complexes, but many of the viruses have not been placed in any of these groups. It is difficult to classify flaviviruses, because they have a wide geographical range and they have diverse arthropod vectors and vertebrate hosts that can be infected (Kuno *et.al.,* 1998). These viruses can infect various cell culture lines and they can also infect a variety of tissue types (Hase *et al.,* 1989; Lindenbach *et al.,* 2007).

The flaviviruses have probably originated from a non-vectored mammalian ancestor. This ancestral virus most probably originated in Africa. Instead of originating sometime after the last glacial period (less than 10 000 years ago) it is estimated that the *Flavivirus* genus originated between 120 000 and 85 000 years ago. After the flaviviruses diverged from its ancestral line, most of them started to diverge about 50 000 years ago into host-vector associations. It is likely that the spread of several

flaviviruses can be linked to the movement of humans out of Africa that took place between 80 000 and 40 000 years ago. Birds and several other animal species contributed to the spread of these viruses (Pettersson, 2013).

1.1.14. Flavivirus structure

Flaviviruses are small, icosahedral, spherical particles 40-50 nm in diameter (Zanotto *et al.,* 1996; Heinz & Allison, 2001, Lindenbach *et al.,* 2007; Pettersson, 2013). They contain an electron dense core (30 nm) that is surrounded by a host-cell derived lipid bilayer (Schlesinger & Schlesinger, 1986; Zanotto *et al.,* 1996; Rodenhuis-Zybert *et al.,* 2010). Mature viral particles contain three major proteins, including capsid (C), envelope (E) and membrane proteins (Heinz & Allison, 2001) These viruses have two or more envelope glycoproteins that surround the nucleocapsid. The nucleocapsid is composed of single-stranded positive-sense genomic RNA (approximately 11 kb long) complexed with multiple copies of small capsid (C) proteins (Chambers *et al.,* 1990; Gaunt *et al.,* 2001; Lindenbach *et al.,* 2007; Pettersson, 2013). The viral surface proteins include the envelope (E) and membrane (M) proteins (Lindenbach *et al.,* 2007). There is a total of 180 copies of the M and E proteins anchored in the envelope (Rodenhuis-Zybert *et al.,* 2010). The E glycoprotein is the main antigenic determinant of the viral particle. These proteins are responsible for binding to specific cell receptors and fusion of the viral particle during viral entry into cells (Heinz & Allison, 2001, Lindenbach, 2007). The M protein is a small fragment of the precursor M protein (PrM) protein. The M protein is produced as the virus matures in the secretory pathways of the cell (Lindenbach *et al.,* 2007). The organization of the flavivirus genome is shown in Figure 1.2.

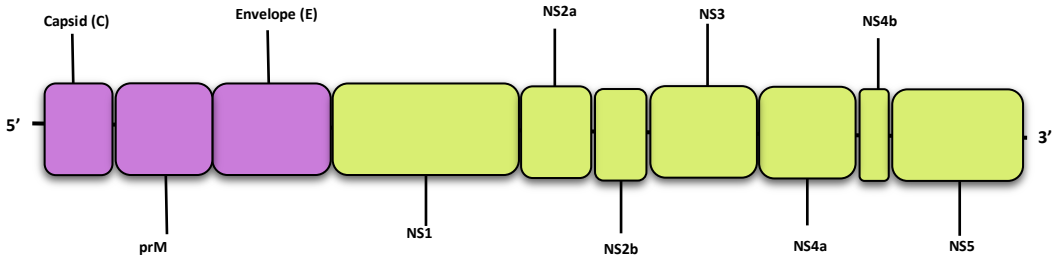

Figure 1.2: Organization of the *Flavivirus* genome showing structural proteins (purple) and non-structural proteins (green).

The viral genome is open to translation directly after fusion happens. Flavivirus genomes differ from cellular RNA in that they do not possess a 3' polyadenylated tail. The genome encodes for a single long open reading frame that is flanked by non-coding regions (NCR's) on both the 3' and 5' ends. The 5' NCR is variable between the different flaviviruses. In the cell the genome functions primarily as the template for RNA replication. The 5' NCR plays a role in the synthesis of positive sense RNA during RNA replication. The genome acts as the mRNA for the translation of all the viral proteins. The RNA genome will also be packaged as genomic material in newly formed viral particles. The organization of genomic RNA is relatively well conserved amongst all the different genera (Lindenbach *et al.,* 2007).

Genomic translation efficiency is of vital importance when looking at flavivirus infectivity. Flaviviruses use several mechanisms to facilitate translation. Translation is cap dependent and it is initiated by ribosomal scanning (Lindenbach *et al.,* 2007). These viruses possess a single open reading frame that is translated into a single large polyprotein (Lindenbach *et al.,* 2007; Rodenhuis-Zybert *et al.,* 2010). This protein contains more than 3 000 amino acids that is cleaved by both host and viral proteases. This polyprotein is post-translationally cleaved into at least ten smaller proteins (Lindenbach *et al.,* 2007). The N-terminal portion of the polyprotein encodes the structural proteins. These proteins include the E, M and C proteins (Lindenbach *et al.,* 2007; Rodenhuis-Zybert *et al.,* 2010). The first protein to be encoded on the 5' end of the flavivirus genome is the C protein followed by the M protein and then the E protein (Schlesinger & Schlesinger, 1986). The remainder of the genome encodes the non-structural proteins. These include NS1, NS2a, NS2b, NS3, NS4a, 2K, NS4b and NS5 (Chambers *et al.,* 1990; Lindenbach *et al.,* 2007; Rodenhuis-Zybert *et al.,* 2010).

Host signal peptidase cleaves the polyprotein between C/prM, prM/E, E/NS1 and 2K/NS4a-NS4b. Virus encoded serine protease is responsible for cleavage between NS2a/NS2B, NS2b/NS3, NS3/NS4a, NS4a/2K and NS4b/NS5 junctions (Lindenbach *et al.,* 2007). Intracellular viral RNA synthesis can begin as early as three hours following infection (Schlesinger & Schlesinger, 1986).

The C protein (about 11kDa) is a highly basic protein. The N- and C terminal ends of the protein contain charged residues with an internal hydrophobic region. This plays a role in membrane association. Nascent C (anchC) contains a C-terminal hydrophobic anchor region. This acts as a signal peptide that facilitates the translocation of prM in the endoplasmic reticulum (ER). The hydrophobic C-terminal is cleaved from the mature C protein by the viral serine protease (Chambers *et al.,* 1990; Lindenbach *et al.,* 2007). The C protein also plays an important structural role as part of the nucleocapsid (Chambers *et al.,* 1990).

The M protein prM (26 kDa) is moved into the ER by the hydrophobic portion of the C protein. Signal peptidase cleavage is delayed, until the viral serine protease cleaves the protein upstream of the signal sequence. This cleavage leads to the formation of the mature C protein. E protein expression also influences the rate of cleavage. The coordinated anchC/prM cleavage delays the processing of structural proteins and the production of viral particles until protease levels are high enough in late infection. The prM protein folds rapidly and it plays an important role in the proper folding of E protein. The C-terminal domains of the prM and E proteins plays a role in the retention of the signals in the ER. prM plays an important role in preventing the E protein from undergoing acid catalyzed rearrangement into the fusogenic form as it moved through the cellular secretory pathways. As the viral particles move through the secretory pathway they are converted from immature to mature forms. This maturation coincides with the cleavage of prM into pr and M fragments (Lindenbach *et al.,* 2007).

Both the prM protein and the pr peptidase act as chaperones stabilizing the E protein. These chaperones aid the E protein in their transit through the secretory pathway, thereby preventing the premature conformational changes of the E protein. Pre-mature changes of the E protein would lead to membrane fusion. As soon as the pr peptide dissociates mature virus are formed that are able to infect new cells (Rodenhuis-Zybert *et al.,* 2010).

The E protein (53 kDa) is the major protein on the surface of flaviviruses. This protein functions in both receptor binding and fusion. The co-expression of the E protein and prM is responsible for proper folding, stabilization in low pH and secretion of the E protein (Lindenbach *et al.* 2007). The E protein is the primary antigen of flaviviruses that elicits neutralizing antibodies (Kaufmann et al., 2009).

The NS1 glycoprotein (46 kDa) is translocated into the ER during the synthesis and cleavage of the E protein by host signal peptidase. Inside the ER an unknown enzyme is responsible for the cleavage at the NS1/NS2a junction. NS1 plays an important role in RNA replication and it is mainly localized to sites of RNA replication. NS1 proteins are retained in infected cells but can localize to the cell surface (Lindenbach *et al.,* 2007). The NS2a and NS2b proteins are small proteins. NS2b is associated with the membrane. NS2b forms a stable complex with NS3 and acts as a cofactor for the NS2b-NS3 serine protease. NS3 (70 kDa) is a large multifunctional protein that plays an important role in processing of the polyprotein and also RNA replication. Flavivirus NS3 encodes an RNA triphosphatase activity that dephosphotylates the 5' end of the genomic RNA before the cap is added. The NS4a and NS4b proteins (16 kDa and 27 kDa respectively) are small hydrophobic proteins. NS4a is associated with NS1 and plays an important role in RNA replication. The NS5 protein is a large protein (103 kDa). This protein has a methyltranferase and RNA dependent RNA polymerase activity. This protein also plays a role in the 5' cap modification (Lindenbach *et al.,* 2007).

1.1.15. Epidemiology, prevalence and transmission of flaviviruses

There are two modes of transmission that involve arthropod vectors. These include the mosquito-borne and the tick-borne flaviviruses (Chambers *et al.,* 1990; Lindenbach *et al.,* 2007). These viruses are distinct, but it appears that they have diverged from a single ancestral line that did not have any vectors (Lindenbach *et al.,* 2007). Isolates have also been found in bats and rodents and these flaviviruses have no known apparent arthropod vectors (Chambers *et al.,* 1990; Pettersson, 2013). The flavivirus group also contains viruses that are not known to be transmitted by vectors such as

Rio Bravo and Modoc viruses. This flaviviruses that are insect specific can only replicate within insect cells and they are not capable of causing disease in humans (Pettersson, 2013). The life cycle of the arthropod-borne flaviviruses involves many important role players including arthropod vectors, vertebrate reservoirs, humans and the environment (Chambers *et al.,* 1990).

Humans are dead-end hosts for arboviral flavivirus infections in natural cycles, but these viruses can still cause significant human and animal diseases within their human hosts (Chambers *et al*., 1990). A vertebrate host is only seen as competent if they can sustain a viraemia at a threshold level for long enough to be infective. In the case of West Nile virus this period is five days. In this period mosquito vectors need to feed on the viraemic host to become infected. Large numbers of infected mosquitoes typically need to feed on hosts to transmit the virus. The transmission of virus from the vertebrate host to the mosquito is dependent on the viraemia of the host. The titers and duration of viraemia is usually higher in birds than in mammals (Garcia-Bocanegra *et al.,* 2010). Humans rarely develop titers that are sufficient to infect mosquitoes and therefore they will not be able to sustain natural infectivity cycles (Castillo-Olivares & Wood, 2004; Garcia-Bocanegra *et al.,* 2010).

1.1.16. Clinical presentation, diagnosis and treatment of flaviviruses

Some of the most important disease-causing agents in this family include: dengue fever virus (DENV), Japanese encephalitis virus (JEV), Murray Valley Encephalitis (MVEV), West Nile virus (WNV), Wesselsbron virus (WSLV), St. Louis encephalitis virus (SLEV), yellow fever virus (YFV) and more recently Zika virus (ZIKV) (Schlesinger & Schlesinger, 1986; Kuno *et.al.,* 1998; Stiasny & Heinz, 2006).

Flavivirus related diseases can be very diverse and complex in their pathological effects (Rice *et al*., 1985). Clinical disease in humans can present as fever, encephalitis or haemorrhagic fever (Chambers *et al.,* 1990).

DENV infections in Asia and the Caribbean have the potential to cause an often-fatal haemorrhagic disease known as dengue shock syndrome. In Asia and the Caribbean, humans are not considered dead-end hosts, with the DENV's having become fixed in *Aedes aegypti*-human transmission cycles. This is also true for ZIKV and CHIKV. JEV,

MVE, WNV and SLEV cause more sporadic outbreaks of disease and periodically cause more explosive epidemics.These infections can be distributed over large geographic areas. Many other flaviviruses are associated with morbidity or mortality in the elderly or in young children (Schlesinger & Schlesinger, 1986).

1.1.17. Emergence and Re-emergence of flaviviruses and alphaviruses

Flaviviruses have shown a recent re-emergence and they are responsible for many emerging and re-emerging human disease outbreaks (Gaunt *et al.,* 2001; Lindenbach *et al.,* 2007). This can be attributed to the fact that traditional mosquito control measures has decreased with limited use of organochlorine pesticides such as DDT which are now frequently replaced with environmentally friendly compounds that are less effective (Lindenbach *et al.* 2007). International travel and urbanization have increased drastically, which leads to the spread of vectors and pathogens to new areas (Lindenbach *et al.* 2007). The re-emergence of these viruses and their spread to new areas emphasize how important they are in a public health perspective (Stiasny & Heinz 2006).

1.2. Problem statement

Mosquitoes are important vectors of many medically significant viruses. Several different species are found in the Bloemfontein area that have the potential to act as vectors of disease. Mosquito surveillance has previously been recorded in the Free State province, however much of this was done 40 to 50 years ago (Edwards, 1941; Muspratt, 1953; McIntosh, 1971; McIntosh, 1973; Jupp, 1978; Jupp *et al.,* 1980; van der Linde *et al.,* 1982; Huang, 1985; van Staden, 1992). These previous researchers did remarkable work with regard to morphological identifications. It is important to know the diversity and density of vector mosquitoes in an area if the circulation of viruses in the area are to be determined. By knowing which species are circulating it becomes easier to try and predict the potential outbreak of viral disease and it is fundamental to help determine control strategies. Although the South African mosquito species have been described morphologically, little information is available on a molecular level. Barcoding sequences are available for many mosquito species, especially for the *cytochrome oxidase c* subunit I. Barcoding data for many of the mosquitoes circulating in the Bloemfontein area is not available on public data bases such as BOLD and GenBank. It is important to link the morphological characteristics of local mosquito populations to their gene sequences. This will aid in the identification of mosquitoes and clarification of the differential roles of species in pathogen transmission. When looking at emerging viral diseases, the presence of viruses and their vectors are as important as environmental factors. Weather conditions such as rainfall and temperature play a large role in the density of mosquito populations, which in turn have an effect on the emergence of diseases carried by these mosquitoes. It is therefore important to consider weather data, mosquito populations and viral emergence. For the most accurate identification of mosquitoes, morphological, molecular and ecological data should be combined (Chan *et al.,* 2014; Erlank *et al.*, 2018).

1.3. Aim

The aim of the study was to identify arboviruses currently circulating in mosquito populations in the Bloemfontein area, Free State province, South Africa.

1.4. Objectives

1. To identify wild caught mosquito species in the Bloemfontein area, Free State province, South Africa, using both conventional morphological characteristics and molecular based methods.
2. To use molecular techniques to identify the viruses circulating in the different mosquito species found in the Bloemfontein area, Free State province, South Africa.

3. To collect meteorological information from weather stations located in proximity to each collection site during the study period.

Chapter 2: Collection and identification of mosquitoes for arbovirus surveillance.

2.1 Introduction

Mosquitoes have a major impact on human and animal health. Effective mosquito surveillance contributes towards developing control strategies for mosquitoes and possibly preventing future outbreaks of disease (Osorio *et al.*, 2008). An effective surveillance program can only be initiated if mosquito species are identified accurately. Mosquito identification was traditionally based on morphological identification using differential dichotomous morphology keys (Edwards, 1941; Gillies & De Meillon, 1968; Rozo-Lopez & Mengual, 2015; Stevenson *et al.,* 2016, Beebe, 2018). Morphological identification of mosquitoes is therefore seen as the gold standard in identification (Chan *et al.,* 2014, Rozo-Lopez & Mengual, 2015; Stevenson *et al.,* 2016, Beebe, 2018). There are, however, a few limitations that are associated with morphological identification.

To identify mosquito specimens effectively detailed keys are very important for ensuring that the differentiation between different species is done without errors. External structures of mosquitoes are complex and the differences between different species in some instances are minute. For these reasons skilled mosquito taxonomists are needed to identify mosquitoes reliably (Wang *et al.*, 2012; Rozo-Lopez & Mengual, 2015; Beebe, 2018).

External characteristics that are used for identification get damaged by the adult mosquito traps that are traditionally used or they can get damaged if they are not stored appropriately (Wang *et al.,* 2012; Beebe, 2018). The scales on the mosquito body are frequently used in many morphological keys, however, these scales tend to rub off easily or get damaged, which has a profound influence on the accurate identification of mosquitoes (Chan *et al.,* 2014; Werblow *et* al., 2016; Beebe, 2018). Specimens are required to be in good condition for accurate identification (Rozo-Lopez & Mengual, 2015).

Within a single collection, a heterogenous group of mosquitoes is normally present. Some mosquito taxa form closely related species complexes that have an overlapping morphology that can make accurate identification difficult (Beebe, 2018).

One of the genes that is widely used for identification of mosquitoes through barcoding, is a region of the mitochondrial gene encoding *cytochrome oxidase c* sub-unit I (COI) (Derycke *et al.*, 2010; Batovska *et al.,* 2016; Beebe, 2018). This gene is popular for species identification, because of the availability of a set of universal primers that can easily amplify an approximately 650 to 700bp region of the 5'end of the gene (Chan *et al.,* 2014; Che *et al.,* 2012). This gene also provides a higher sequence variation at inter-species rather than intra-species level (Chan *et al.,* 2014). The COI gene is present in hundreds of copies per cell and the region lacks introns. This gene exhibits the slowest rate of change in amino acid sequence than any other mitochondrial gene. This increases the resolution of molecular identification and it aids in primer design (Cywinska *et al.,* 2006*).*

The COI gene has limitations associated with its efficiency as a barcoding sequence for the identification of mosquito species. It has been documented that the COI barcode has failed to identify certain species of *Culex* and *Anopheles* (Chan *et al.,* 2014). Certain *Ochlerotatus* species could also not be differentiated using the COI barcoding sequence (Chan *et al.,* 2014). The success of specific genetic barcodes is also dependent on the availability of representative sequences with which to compare them. If there are not enough reference sequences available for comparison, the barcoding approach will fail (Chan *et al.,* 2014). Other regions frequently used for DNA barcoding include the inter spacer region two (ITS2), cytochrome oxidase b, 12SrRNA and nicotinamide adenine dinucleotide dehydrogenase (NADH) (Chan *et al.,* 2014).

Mosquitoes are slender insects and many parts of their bodies are very delicate. Mosquitoes are very abundant and can be found nearly everywhere in the world. Although they frequent temperate and tropical regions, they can also be found in the areas surrounding the Arctic Circle. Mosquitoes are not found in Antarctica and certain isolated islands (Service, 2004; Harbach, 2007). Mosquitoes will therefore be found anywhere where it is not permanently frozen (Clements, 1992). Various ecological factors play a crucial role, not only on mosquito behaviour, but also on the vector

capacity of mosquitoes. These include humidity, rainfall, temperature, wind speed, vegetation, soil type, quality of water bodies and elevation (Mattingly, 1969).

Mosquitoes are important vectors of disease, and knowledge of mosquito species circulating in the Bloemfontein area has not been well documented in recent years. Most of the mosquito species in the area have not previously been molecularly typed and there is limited genetic data available on GenBank or the Barcode of Life Database (BOLD) to supplement conventional morphological identification. In this study, mosquitoes collected at three sites in and around Bloemfontein were identified using conventional morphological identification. Selected representative species were subsequently assayed using molecular methods and barcoding primers targeting a region of the COI gene.

2.2 Materials and methods

2.2.1 Study area

Mosquitoes were collected from three different sampling sites in and around the Bloemfontein area, Free State, South Africa. The three sampling sites were identified based on locality, the presence of a permanent water body or, in the case of the urban site, a water retainer, and an active bird population. The three sampling sites were as follows:

Krugersdrift Dam (28º 88’ 36” S; 25º 95’ 83” E), which was selected to represent a rural environment. The Krugersdrift Dam is situated about 50 km from Bloemfontein and it is surrounded by farmland. The only human disturbances that are located near the dam are a tar road and the occasional fisherman. This area has an active water bird population and vervet monkeys (*Cercopithecus aethiops*) can be found in the wooded areas surrounding the dam. This area was selected as a sampling site because it has the potential to be a site for possible avian reservoir hosts of flaviviruses and alphaviruses. The Krugersdrift Dam is an artificial dam that flows into the Modder River. It is a permanent water body. In recent years, the water levels have receded,

but due to increased rainfall in the summer of 2017 water levels in the dam have increased. The dam is surrounded by a short grass field with Acacia bush on the outer fringes.

The Free State National Botanical Gardens in Bloemfontein (29º 05' 26" S; 26º 21' 28" E) was selected as a peri-urban environment. A peri-urban environment is any environment that forms the boundary between an urban and rural area. This area functions perfectly as a peri-urban area, because it has large expanses of grassland, but it is also in an area that has a fast-growing human population with large new expanses of human settlements. It therefore functions as a fringe environment between urban and rural areas. This area is a permanent vlei area that has an active bird population. Within the gardens there is an ephemeral pan that provides an excellent breeding area for mosquitoes. The pan also attracts a diverse water bird population.

Bloemfontein Zoo (29º 19' 44" S; 26º 05' 56" E) was selected as a sampling site that represented an urban environment. The zoo is close to the Loch Logan Spring and it has both primate and bird reservoir hosts. This environment was considered as urban, because it is surrounded by human settlement and it has high human traffic. It also has several different artificial water reservoirs that have the potential to act as breeding sites for mosquitoes.

2.2.2 Sample collection and morphological identification of adult mosquitoes using entomological keys.

Mosquitoes were collected using Shannon traps and CDC light traps baited with dry ice. Dry ice was chosen as an attractant, because it gives off carbon dioxide when evaporating. Carbon dioxide acts as an olfactory attractant to mosquitoes and it acts as one of the primary cues for mosquitoes when they detect prey. Shannon traps are large tent traps. These tent traps are open at the bottom. Most mosquitoes fly closely to the ground at night and will therefore enter the trap from the bottom. Once in the trap, mosquitoes tend to fly upwards and rest near the top of the trap. During the day, mosquitoes avoid direct sun and wind, so tend to rest on vegetation that is close to the ground. This prevents desiccation and unnecessary expenditure of energy.

Mosquitoes in the Shannon trap will therefore fly downwards at dawn and escape the trap, making it imperative that the trapped mosquitoes are collected before sunrise. Due to this behaviour all mosquito catches were done between 17H00 and 05H00. The time of 17H00 allows for the capture of mosquitoes that are diurnal, nocturnal or active in the crepuscular periods. The mosquitoes were collected from the tents before 05H00 to prevent mosquitoes from escaping the tent.

The CDC light trap works on a suction basis where mosquitoes get sucked into a netting container. The CDC light trap is fitted with a light source and it is baited with dry ice. The CDC light traps were only used for December. It was found that the trap damaged the mosquitoes substantially. The battery also did not work efficiently when there was slight rainfall. The mosquitoes from the Shannon trap and the CDC light trap were both collected using a battery-operated vacuum aspirator.

Mosquito collections were done at intervals between December 2016 and April 2017, with focus placed on the periods of February 2017 to April 2017, the peak period for arbovirus circulation in South Africa.

The traps at the Krugersdrift Dam were placed ten metres away from the banks of the dam in an area that contains short grass. The area directly surrounding the trapping site does not have any trees or brush. The traps in the Free State National Botanical Gardens were placed three metres away from the banks of the pan. The traps were set up in an open area of short grass. The mosquito traps in the Bloemfontein Zoo were placed centrally between the enclosures of several species of primates and birds containing water retainers. The traps were set up in an open area with short grass. The trap was placed ten metres away from a stagnant pool of water.

Upon collection, the mosquitoes were placed in specimen collection containers containing RNAlater® (Sigma-Aldrich, Missouri, USA) to preserve viral RNA. The mosquitoes were transported in these containers to the Division of Virology, Faculty of Health Sciences, University of the Free State. All mosquitoes were transported to the laboratories following the correct protocols. The transport protocol can be seen in Appendix 5.6.

All mosquito samples were placed at -20°C in RNAlater® (Sigma-Aldrich, Missouri, USA). Mosquitoes were identified within of two weeks after collection, because there

was a tendency for the mosquitoes to lose scales in the RNAlater®. The frozen specimens were identified using the entomological keys that are specific to mosquitoes found in southern Africa. Two different sets of keys were used for identification. All the mosquitoes in the Culicinae sub-family were identified using the entomological keys of Jupp (1996). The mosquitoes in the sub-family Anophelinae were identified using entomological keys by Gillies & Coetzee (1987). These two sub-families are quite easy to identify by observation; therefore no keys were used to identify the mosquitoes to sub-family level.

Mosquitoes were kept on ice during morphological identification. They were placed on a clear sterile petri dish to ensure easy movement of the specimen without excessive handling. Mosquitoes were carefully observed under different magnifications on a Nikon MicroLite FV1000 light microscope (10-1 000 times magnification (Massachusetts, USA) to get an overall view of the morphological structures that define each species. After each specimen was identified, the legs and wings were removed using sterile wooden toothpicks. Work was performed in a class 2 biosafety cabinet in a biosafety level 2 laboratory. The head, thorax and abdomen of each specimen were placed in a clean 1.5 ml micro-centrifuge tube and stored in RNAlater® (Sigma-Aldrich, Missouri, USA) at -20˚C. All tubes were clearly marked with a vector-borne disease fieldwork number (VBDF), collection date, collection site and species name. The VBDF is a number that is associated with the collection date and collection site and can therefore be used to quickly retrieve information for the mosquito specimen. The legs and wings of each specimen were placed in a separate micro-centrifuge tube. All legs and wings were stored at -20°C until downstream reactions were performed. DNA was extracted from the legs for genetic barcoding.

After identification of the species, the bodies of mosquitoes identified as the same species were pooled for arbovirus studies. The pools ranged in size from 1-5 mosquitoes depending on how many mosquitoes were identified per species. The pools were placed in 1.5 ml micro-centrifuge tubes and clearly marked with the collection site, collection date, collection number and species name. These mosquito pools were stored in RNAlater® (Sigma-Aldrich, Missouri, USA) at -20°C.

2.2.3 Molecular identification of mosquitoes

2.2.3.1 DNA extraction

DNA was extracted from mosquito specimens using the QIAGEN Blood and Tissue Minikit from Qiagen (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. The experimental procedure was validated using a single mosquito specimen that was positively identified as *Aedes aegypti* using entomological keys. For optimisation of the procedure, the DNA extraction procedure was repeated with mosquito leg and head. This was done to determine that a mosquito leg can be successfully used as the starting material for DNA extraction. The extracted DNA was diluted 1:100 and 1:1 000 to determine that small sample sizes can be used for species identification. A region of the COI gene was amplified as described below and the PCR amplicon purified, and the nucleotide sequence of the amplicon determined using Sanger sequencing. For subsequent identifications only the leg was used for extraction of DNA.

Representative mosquito specimens were selected from species groups that were identified through morphological identification. These included two specimens of *Ochlerotatus harrisoni*, one specimen of *Ochlerotatus juppi*, one specimen of *Mansonia uniformis*, one specimen of *Aedes aegypti*, sixteen specimens of *Anopheles squamosus* and four specimens of *Culex theileri*. The two *Ochlerotatus* species were known previously as *Aedes* (*Ochlerotatus*) *harrisoni* and *Aedes* (*Ochlerotatus*) *juppi*, but in this study they will be referred to by their new classification, *Ochlerotatus harrisoni* and *Ochlerotatus juppi*. The sub-genes Ochlerotatus was elevated to genus level in 2000. It is accepted to use the Ochlerotatus as a genus name (Wilkerson *et al.,* 2015).

Briefly, each mosquito leg was initially homogenized in liquid nitrogen using a disposable micro-pestle (Sigma-Aldrich, Kenilworth, New Jersey, USA). The homogenate was lysed by the addition of 90 µl of buffer ATL and 10 µl of proteinase K that was included in the kit. The sample was incubated at 56°C overnight and centrifuged the following day at 14 000xg for 15 seconds using a Spectrafuge 16M centrifuge (Labnet™ International Inc., Woodridge, New Jersey, USA). A total volume

of 100 µl of AL buffer was added to the sample and incubated at 70°C for 10 minutes. A total volume of 100 µl of 70% ethanol was added to the sample, vortexed and centrifuged at 14 000xg for 10 seconds. The mixture was transferred to a QIAamp mini spin column and centrifuged at 6 000xg for one minute. Wash steps were carried out using AW1 and AW2 buffers provided in the kit to remove residual contaminants. The DNA was eluted in 50 µl of AE buffer. For short term storage the DNA was stored at -20°C and for long term storage an aliquot was frozen at -80°C.

2.2.3.2 PCR amplification of mosquito DNA

DNA, extracted from each mosquito leg, was used as the template for a PCR reaction. The PCR was performed using a primer pair that amplifies a region of the *Cytochrome oxidase c* I mitochondrial gene (Folmer *et al*., 1994). The primer sequences are provided in Table 2.2.1.

The PCR was performed using Go Taq®G2 Hot Start Polymerase (Promega, Madison, Wisconsin, USA) according to manufacturers' instructions. The reaction components are provided in Table 2.2.2. For the negative control a 1 µl aliquot of nuclease free water was added instead of DNA template. The reactions were cycled on a Proflex PCR System thermocycler (Life Technologies, Carlsbad, California, USA) using the following cycling conditions: initial denaturation at 95°C for two minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds and elongation at 72°C for one minute and a final elongation at 72°C for five minutes.

Table 2.2.1: Nucleotide sequence of barcoding primers used to amplify a 715 bp region of the *cytochrome oxidase c* sub-unit I gene for genetic identification of mosquitoes (Folmer *et al.,* 1994).

Primers	**Nucleotide sequence 5' to 3'**	**Forward /Reverse**	***T_m**	**Amplicon size**
HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	Forward	65°C	~715 bp
LCO1490	GTCAACAAATCATAAAGATATTGG	Reverse	58°C	

*T_m calculated using Biomath Calculator from Promega (www.**promega**.com/a/apps/**biomath**/?calc=tm).

Table 2.2.2: PCR reaction components used to amplify a region of the *cytochrome oxidase c* sub-unit I gene of field caught mosquitoes.

PCR components	**Final volume**	**Final concentration**
5x Green Go Taq® flexi buffer	10 µl	1X
$MgCl_2$ solution, 25 mM	4 µl	2 mM
PCR nucleotide mix, 10 mM each	1 µl	0.2 mM
HCO (forward primer) (20 pmol/µl)	1 µl	0.4 µM
LCO (reverse primer) (20 pmol/µl)	1 µl	0.4 µM
Go Taq® G2 Hot Start Polymerase (5 U/µl)	0.25 µl	1.25 U
DNA Template	1 µl	-
Nuclease free water (NFW)	31.75 µl	-
Total	**50 µl**	-

The PCR amplicons were separated by electrophoresis of a 4 µl aliquot of PCR amplicon using a 1% Seakem® LE agarose gel (Lonza, Maine, USA), prepared in Tris-acetate-EDTA (TAE) buffer (pH 8.0). A total volume of 3µl of O'GeneRuler™ 100 bp DNA ladder SM 1173 (ThermoFischer Scientific, Massachusetts, USA) comprising of DNA fragments from 100 to 10 000 bp was used to estimate the size of PCR amplicons. A 3 µl aliquot of O'GeneRuler™ was mixed with 1 µl of 4 000x GelRed® loading dye (Biotium, Fremont, California, USA). Agarose gel electrophoresis was performed using BioRad PowerPac Basic Systems (BioRad, California, USA) at 100V, 400A for 35 minutes. PCR amplicons were visualised using a UV transilluminator (UVItec, Cambridge, UK).

2.2.3.3. Purification of PCR amplicons

Wizard ®SV Gel and PCR Clean-Up System was used for purification of PCR amplicons according to manufacturers' instructions (Promega, Madison, Wisconsin, USA). This system is based on the ability of the DNA to bind to silica membranes in the presence of chaotropic salts and removes excess nucleotides, primers and enzymes. Following electrophoresis, the band of interest was excised from the agarose gel and transferred to a 1,5 ml micro-centrifuge tube. The gel slice was dissolved in 1 mg/ml membrane binding solution in a dry heating block at 56°C. After

incubation, the melted gel mixture was transferred to a mini-column system and incubated at room temperature for one minute. The mini-column system was centrifuged at 14 000xg for one minute. Isolated PCR amplicon was washed twice using membrane wash solution to remove excess nucleotides. The DNA was eluted in a 30µl aliquot of NFW, centrifuged at 14 000xg for one minute and stored at -20°C for downstream application.

The purification of the PCR amplicons was confirmed by electrophoresis of a 4 µl aliquot of purified PCR amplicon using a 1% Seakem® LE agarose gel (Lonza, Maine, USA), prepared in TAE buffer. The agarose gel electrophoresis was performed as described in section 2.2.3.2.

DNA concentration was determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Illinois, USA). The purity was determined from the 260nm:280nm ratio of absorbance.

2.2.3.4. DNA sequence determination

Determination of the nucleotide sequence of the amplicon was performed using the BigDye® Terminator V3.1 sequencing ready reaction kit according to manufacturers' instructions (Applied Biosystems, Foster City, CA, USA). Sequencing reaction components are shown in Tables 2.2.3 and 2.2.4. Reactions were cycled using the following cycling conditions: initial denaturation, 96°C for one minute, followed by 25 cycles of 96°C for 10 seconds, annealing, 50°C for five seconds and extension, 60°C for four minutes. Lastly samples were held at 4°C indefinitely.

Table 2.2.3: Sequencing reaction components used for the sequencing of mosquito legs.

Components	**Volume**
Ready reaction	1 µl
HCO2198 (0.8 pmol/µl) / LCO1490 (0.8 pmol/µl)	4 µl
Dilution buffer	2 µl
Template DNA	1-3 ng
NFW	to a total of 10 µl

Table 2.2.4: Control sequencing reaction mix used as the control in sequencing reactions with mosquito legs.

Components	**Volume**
Ready reaction	1 µl
Control sequencing primer(0.8 pmol/µl) (M13)	4 µl
Dilution buffer	2 µl
Control sequencing plasmid (pGem-3z(f)t)	1 µl
NFW	2 µl
Total control reaction	**10 µl**

For post reaction clean-up, an EDTA/ethanol precipitation was used. A solution of 0.5 M EDTA (pH 8.0) was diluted to 125mM with NFW. A 5 µl aliquot of 125 mM EDTA and 60µl absolute ethanol were added to a 1.5ml micro-centrifuge tube. The sequence reaction volume was adjusted to 20 µl reaction by adding 10 µl NFW. The diluted sequencing reaction was added to the tube containing 125 mM EDTA and 60 µl absolute ethanol. The tube was vortexed for five seconds. Precipitation was allowed by leaving the reaction at room temperature for 15 minutes. The samples were centrifuged at 14 000xg for 10 minutes at 4°C. The supernatant was completely aspirated without disturbing the pellet. A volume of 500 µl of 70% ethanol was added to each tube. The reaction tubes were centrifuged at 14 000xg for 10 minutes at 4^0C. The supernatant was completely aspirated without disturbing the pellet. The reaction tubes were incubated at 37°C for two hours until completely dry. Lastly the samples were stored at 4°C in the dark until submission for electrophoresis.

2.2.3.5. Genetic identification of mosquito species

Nucleotide sequence data from positive PCR amplicons were edited using Chromas Pro version 1.6 and aligned using Clustal Omega version 1.2.1. The mosquito species identified through morphological means were confirmed through comparison with nucleotide sequence data retrieved from GenBank and by BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

The forward and reverse sequences for each mosquito species were assembled into a contiguous sequence using Chromas Pro version 1.6. The ends of these combined

sequences were trimmed and the chromatograms were reviewed manually to edit misread bases.

2.2.4. Collection of meteorological data

Meteorological data for the study period was obtained for the Bloemfontein area from the National Weather Services'. The data included daily rainfall and daily minimum and maximum temperatures. Data were collected from the three weather stations located closest to the three sampling sites. The Glen Agricultural College (28° 94' 20" S; 26° 32' 50" E) is located near the Free State National Botanical Gardens. This station is 1297 m above sea level. Weather data were available from the 17th of June 2004. Bloemfontein City Centre weather station (29° 12' 00" S; 26° 18' 70' E) is near the Bloemfontein zoo and it is 1406 m above sea level. Weather data were available from the 1st of January 2002. West Bloemfontein (29° 10' 30" S; 26° 29' 80"E) weather station is located near the Krugersdrift Dam and it is 1340 m above sea level. Data at this weather station were available from the 1st of January 2002.

2.3. Results

2.3.1 Sample collection and morphological identification of adult mosquitoes using entomological keys.

A total number of 318 mosquito specimens were collected from the three sampling sites in the Bloemfontein area and all of them were included in this study. A total of 11 mosquitoes were caught with the CDC light trap. The remaining 307 mosquitoes were caught using the Shannon trap. On average the number of mosquitoes caught in the CDC light trap per night was three mosquitoes (2,75) and the Shannon trap caught an average of nine (9,33) mosquitoes per night. To get a more accurate result regarding trapping success more collections need to be done using the CDC light trap. Mosquitoes were identified in two major mosquito sub-families, the Anophelinae and Culicinae, belonging to four different genera including *Anopheles, Aedes, Culex* and *Mansonia* and thirteen different species groups as follows:

Genus *Aedes* Meigen. This group forms part of the tribe Aedini. Within this genus three sub-genera were identified including, *Neomelaniconion* Newstead*, Ochlerotatus* Lynch Arribalzaga and *Stegomyia* Theobald (Jupp, 1996). In the *Neomelaniconion* sub-genus one specimen could not be identified to species level because it was damaged, and one specimen was identified as *Aedes* (*Neomelaniconion*) *unidentatus* McIntosh. Within the *Ochlerotatus* group sub-genus, two species were identified, including *Ochlerotatus juppi* McIntosh and *Ochlerotatus harrisoni* Muspratt*.* In the sub-genus *Stegomyia* only *Aedes* (*Stegomyia*) *aegypti* (Linnaeus) was identified.

Genus *Anopheles* Meigen. This group contains 488 spesies. This genus is divided into three genera, including: *Anopheles*, *Bironella* and *Chagasia* and eight sub-genera. These include *Cellia, Anopheles, Nyssorhynchus, Stethomyia, Baimaia, Christya, Kerteszia* and *Lophopodomyia (*Harbach, 2008, Foster, 2017*)*. The main species identified in the Bloemfontein area is *Anopheles squamosus* Theobald. Four specimens were badly damaged and could not be identified to species level, only to genus.

Genus *Culex* Linnaeus. This group forms part of the tribe Culicini. Within the group two sub-genera were identified, including *Culex* Linnaeus and *Lutzia* Theobald (Jupp, 1996). In the sub-genus *Culex* three species were identified, including *Culex* (*Culex*) *pipiens* Linnaeus, *Culex* (*Culex*) *theileri* Theobald and *Culex* (*Culex*) *univittatus* Theobald. One specimen could not be identified to species level but had characteristics that suggest it possibly being *Culex* (*Culex*) *terzii/vansomereni* Edwards. One specimen was identified as sub-genus *Lutzia,* species *Culex (Lutzia) tigripes* De Grandpre & De Charmoy.

Mansonia uniformis was the only species found in the genus *Mansonia*. This specimen was found in the Free State National Botanical Gardens.

A total of 32 mosquito specimens were collected at the Krugersdrift Dam during January, March and April 2017. A total of 228 mosquitoes were captured between December 2016 – April 2017 at the Free State National Botanical Gardens (FSNBG). A total of 58 mosquito specimens were collected at the Bloemfontein Zoo between December 2016 – April 2017.

The total number of mosquitoes collected in the Bloemfontein area from December 2016 – April 2017 and the morphological identification is shown in Table 2.3.1.

Table 2.3.1: Morphological identification, date of collection, collection site and number of mosquitoes caught over the study period December 2016 – April 2017.

Morphological identification	Date of collection	Collection site	Number of specimens collected	Collection Method
Anopheles squamosus	10 December 2016	Bloemfontein zoo	1	Shannon trap
Anopheles squamosus	10 December 2016	Bloemfontein zoo	1	CDC light trap
Ochlerotatus harrisoni	10 December 2016	Bloemfontein zoo	2	CDC light trap
Culex theileri	10 December 2016	Bloemfontein zoo	6	Shannon trap
Culex theileri	11 December 2016	FSNBG*	7	CDC light trap
Culex univittatus	11 December 2016	FSNBG	1	CDC light trap
Culex pipiens	11 December 2016	FSNBG	1	Shannon trap
Culex theileri	28 January 2017	Bloemfontein zoo	1	Shannon trap
Ochlerotatus juppi	29 January 2017	FSNBG	1	Shannon trap
Culex theileri	29 January 2017	FSNBG	1	Shannon trap
Anopheles squamosus	30 January 2017	Krugersdrift Dam	6	Shannon trap
Culex theileri	27 February 2017	Bloemfontein zoo	6	Shannon trap
Culex tigripes	27 February 2017	Bloemfontein zoo	1	Shannon trap
Culex spp.	27 February 2017	Bloemfontein zoo	1	Shannon trap
Ochlerotatus juppi	27 February 2017	Bloemfontein zoo	3	Shannon trap
Ochlerotatus harrisoni	27 February 2017	Bloemfontein zoo	6	Shannon trap
Anopheles squamosus	28 February 2017	FSNBG	24	Shannon trap
Anopheles squamosus	17 March 2017	Bloemfontein zoo	12	Shannon trap

Anopheles spp[1]	17 March 2017	Bloemfontein zoo	2	Shannon trap
Culex theileri	17 March 2017	Bloemfontein zoo	4	Shannon trap
Aedes (Neomelaniconion) spp.	17 March 2017	Bloemfontein zoo	1	Shannon trap
Aedes juppi	17 March 2017	Bloemfontein zoo	2	Shannon trap
Culex univittatus	17 March 2017	Bloemfontein zoo	3	Shannon trap
Anopheles squamosus	18 March 2017	FSNBG	145[#]	Shannon trap
Culex theileri	18 March 2017	FSNBG	5[#]	Shannon trap
Aedes unidentatus	18 March 2017	FSNBG	2	Shannon trap
Culex theileri	19 March 2017	Krugersdrift Dam	13	Shannon trap
Anopheles squamosus	19 March 2017	Krugersdrift Dam	2	Shannon trap
Anopheles squamosus	01 April 2017	Bloemfontein zoo	2	Shannon trap
Culex theileri	01 April 2017	Bloemfontein zoo	4	Shannon trap
Anopheles squamosus	02 April 2017	FSNBG	30	Shannon trap
Aedes aegypti	02 April 2017	FSNBG	1	Shannon trap
Mansonia uniformis	02 April 2017	FSNBG	1	Shannon trap
Culex pipiens	02 April 2017	FSNBG	1	Shannon trap
Culex theileri	02 April 2017	FSNBG	6	Shannon trap
Anopheles spp.	02 April 2017	FSNBG	2	Shannon trap
Anopheles squamosus	03 April 2017	Krugersdrift Dam	11	Shannon trap
Total			318	

[*]FSNBG is the abbreviation for the Free State National Botanical Gardens.

[#] A single specimen of each of these collections was stored intact as a reference specimen.

The CDC light trap was only used for collections in December 2016. It was found that specimens captured in these traps have a lot more damaged than the ones in the net trap. The CDC light trap also had problems with its battery when there was slight rainfall. The trap was also set up in January and February, but no mosquitoes were captured.

Krugersdrift Dam

The Krugersdrift Dam had a relatively low mosquito diversity compared to the other sampling sites. Only two species were collected at this site, *Anopheles squamosus,* the most abundant group, making up 19/32 (59%), and *Culex theileri,* 13/32 (41%) of the total number of mosquitoes collected (Figure 2.3.1.). In contrast, eight and six different species were collected at the FSNBG and the zoo respectively.

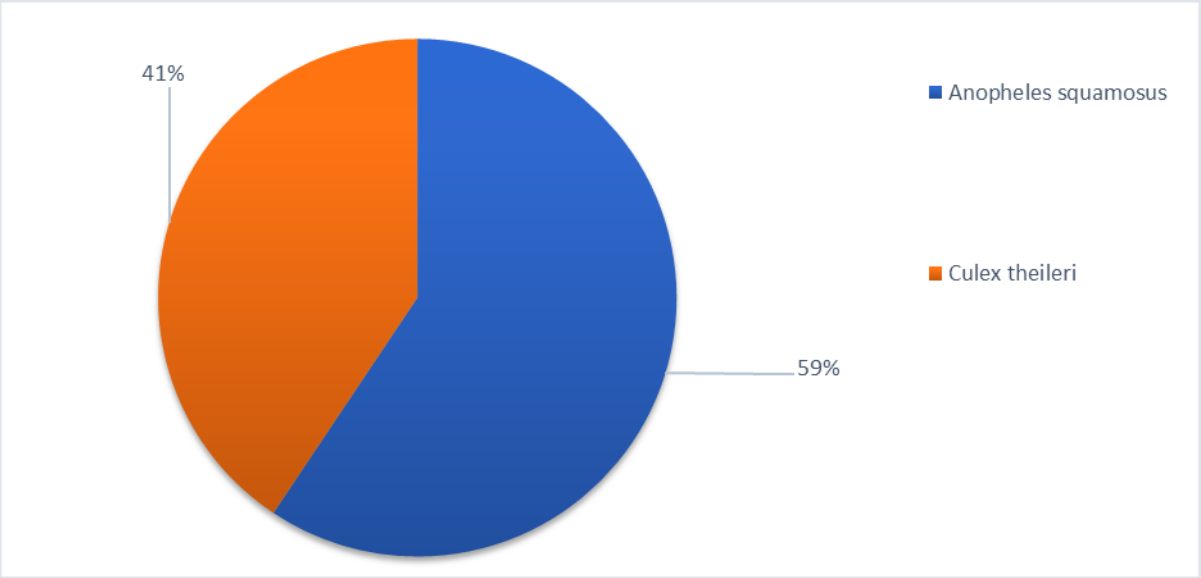

Figure 2.3.1: Species diversity of mosquitoes collected from the Krugersdrift Dam from January 2017-April 2017.

The diversity of mosquito species from the Krugersdrift Dam over the sampling period is shown in Figure 2.3.2. The majority of mosquitoes were collected in March 2017. Mosquito collections were not possible in December 2016 and February 2017 due to unforeseen circumstances.

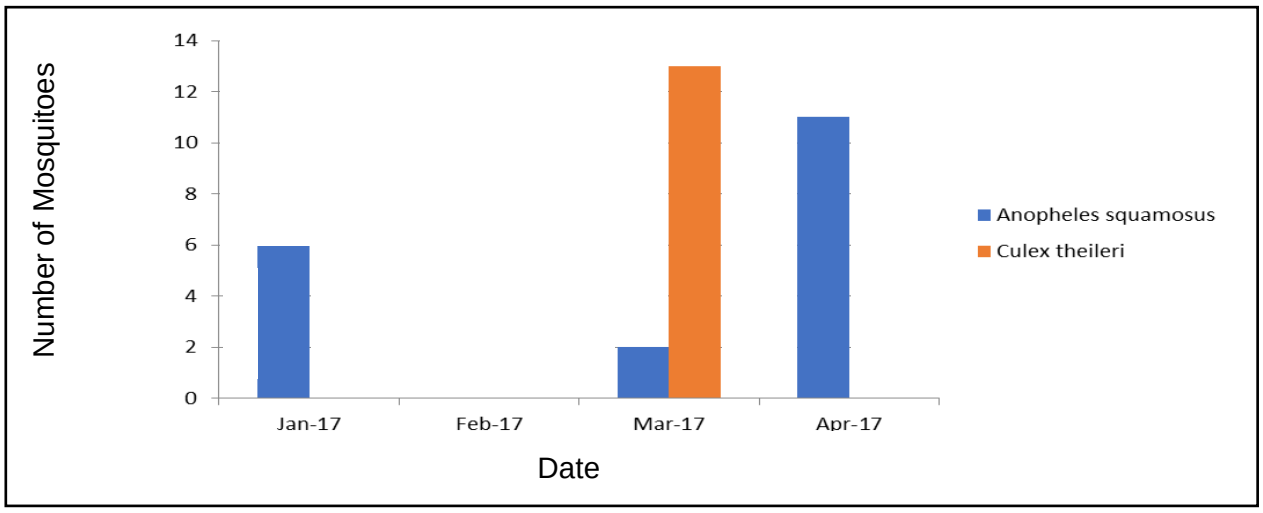

Figure 2.3.2: The temporal distribution of mosquitoes caught at the Krugersdrift Dam from January 2017– April 2017.

Free State National Botanical Gardens

The majority of mosquitoes collected in the study were from the FSNBG. Eight different species were caught during the sampling period (Fig. 2.3.3). *Anopheles squamosus* was the most abundant group, making up 199/228 (87%) of all mosquitoes collected in this area. Of the remaining mosquitoes, 19/228 (8%) were identified morphologically as *Culex theileri.*

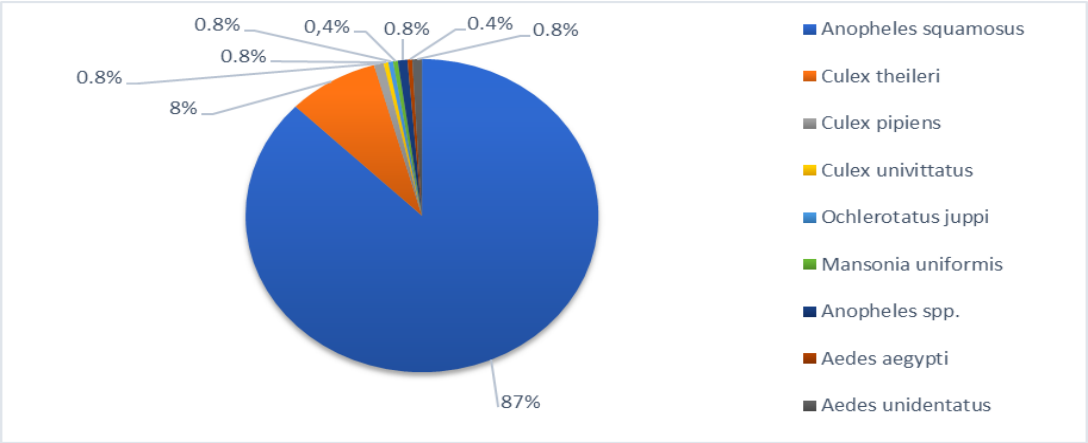

Figure 2.3.3: Species diversity of wild caught mosquitoes from the Free State National Botanical Gardens from December 2016-April 2017.

The diversity of mosquito species collected at the Free State National Botanical Gardens over the sampling period is shown in Figure 2.3.4. Most of the mosquitoes, 152/228, were collected in March 2017. In contrast 2/228 were collected in January 2017. The lowest diversity of mosquitoes was seen in February 2017 with only one species identified.

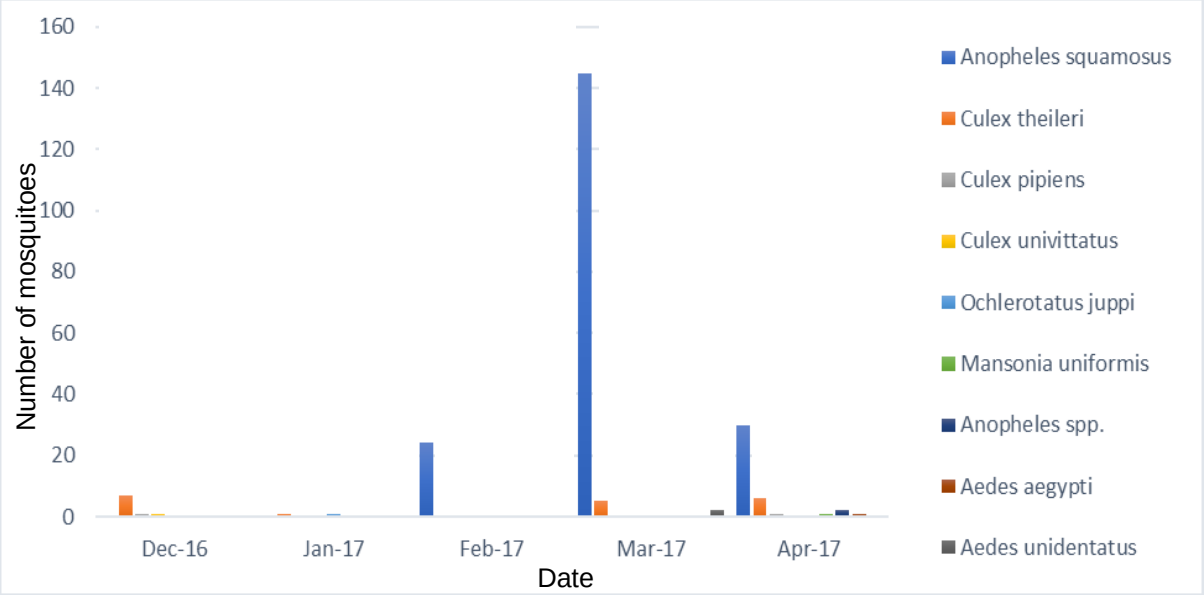

Figure 2.3.4: The temporal distribution of mosquitoes caught in the Free State National Botanical Gardens from December 2016 – April 2017.

Bloemfontein Zoo

The diversity of species that were collected at this site is shown in Figure 2.3.5. Six different species were identified during the sampling period of which *Culex theileri* was the most abundant group, making up 21/58 (36%) of all mosquitoes collected. *Anopheles squamosus* was the second largest species group making up a total of 16/58 (27%) of the total number. In the case of one *Aedes (Neomelaniconion)* spp. and three *Anopheles* spp., the specimens were damaged in a way that made morphological identification to species level very difficult and therefore these specimens were only identified to genus.

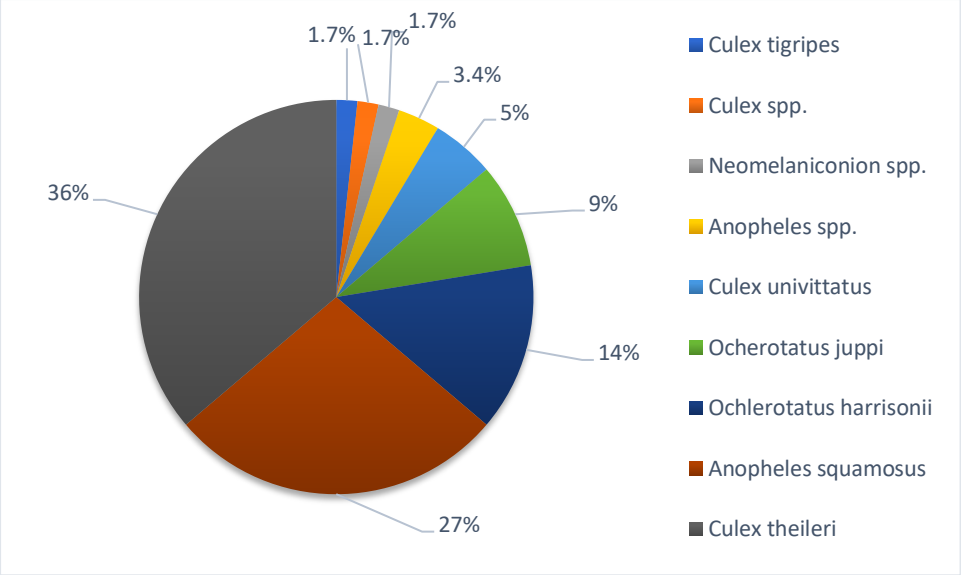

Figure 2.3.5: Species distribution of wild caught mosquitoes from the Bloemfontein Zoo from December 2016-April 2017.

The distribution of mosquito species collected at intervals at the Bloemfontein Zoo over the sampling period is shown in Figure 2.3.6. The highest number and diversity of mosquito species were collected in March 2017. Six different species of mosquito were collected during March. In January 2017, only one mosquito species was collected.

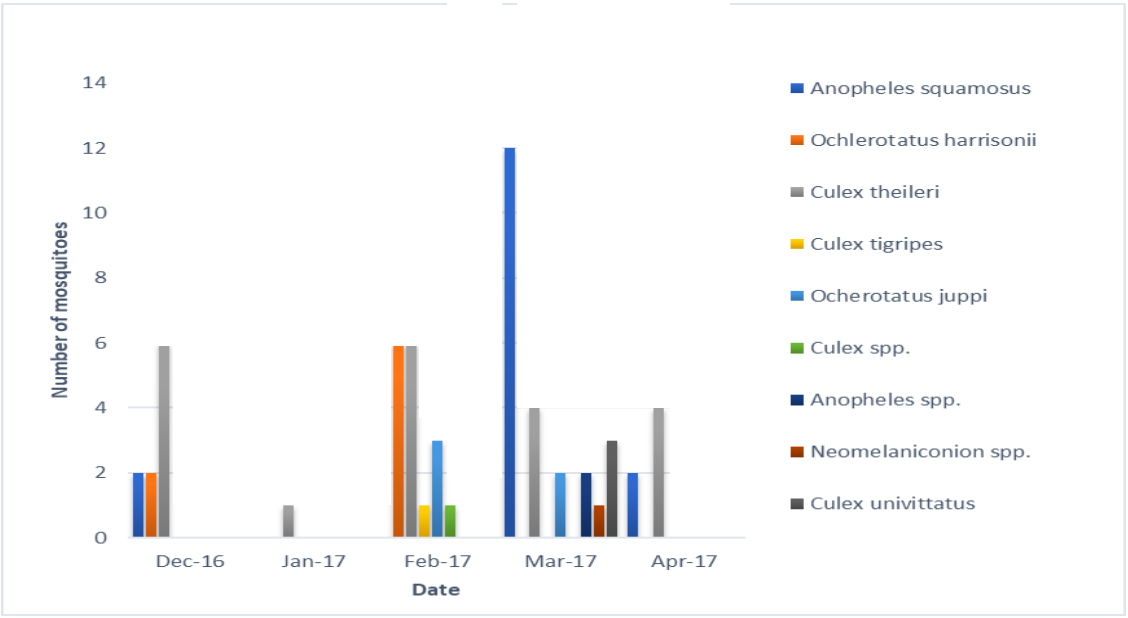

Figure 2.3.6: The species distribution of mosquitoes caught in the Bloemfontein Zoo from December 2016 – April 2017.

The combined mosquito species diversity for the Bloemfontein area can be seen in Figure 2.3.7. A total of ten different species were identified in the area. *Anopheles squamosus* was the largest species group making up a total of 234/318 (74%) of the total number of mosquitoes collected. *Culex theileri* was the second largest group with a total of 53/318 (17%) of the mosquitoes caught.

Bloemfontein Area

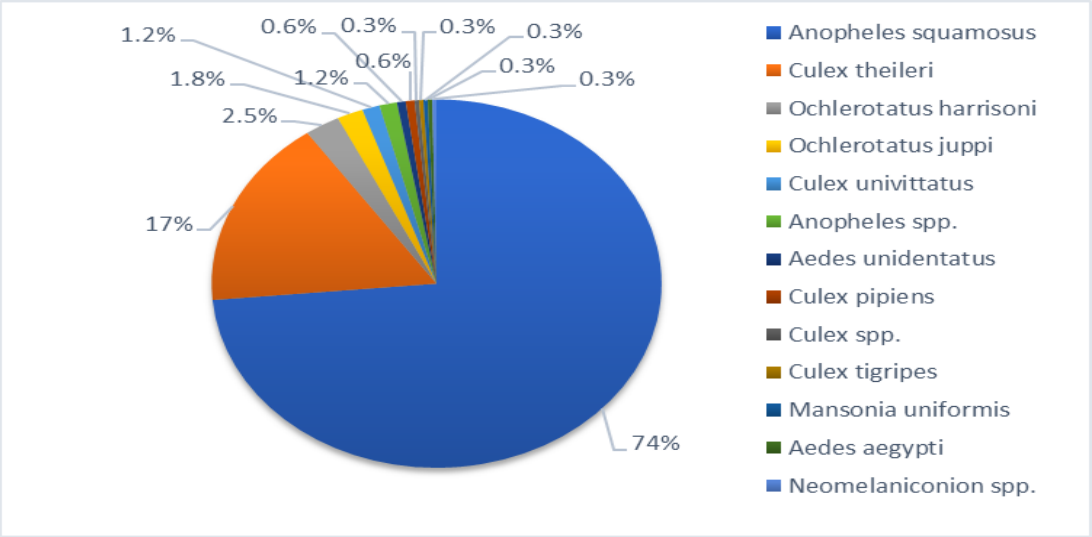

Figure 2.3.7: Species diversity of wild caught mosquitoes from the Bloemfontein area from December 2016-April 2017.

The temporal distribution of mosquito species collected in the Bloemfontein area between December 2016 to April 2017 is shown in Figure 2.3.8. The highest number and diversity of mosquito species were collected in March 2017. From the collection data it is clear that mosquito activity starts to increase from February, with a peak period of activity in March.

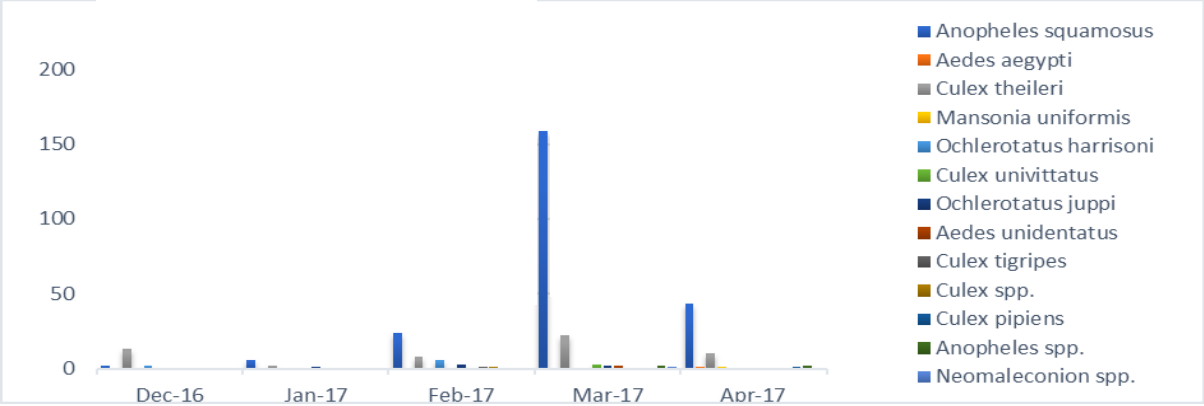

Figure 2.3.8: The species distribution of mosquitoes caught in the Bloemfontein Area from December 2016 – April 2017.

2.3.2. Molecular identification of mosquito species

The DNA extraction protocol was initially optimised using DNA extracted from a mosquito, collected in this study that was morphologically identified as *Aedes aegypti*. The DNA extraction technique was performed using the head of the mosquito and a single leg of the mosquito. A PCR was performed using the HCO2198 and LCO1490 primer pair and an aliquot of the PCR products were separated and visualised by electrophoresis. The gel electrophoresis results can be seen in Figure 2.3.9. DNA bands with predicted size of 715bp were detected. The results for the mosquito head and legs confirm that DNA extracted from a single mosquito leg was suitable for the technique.

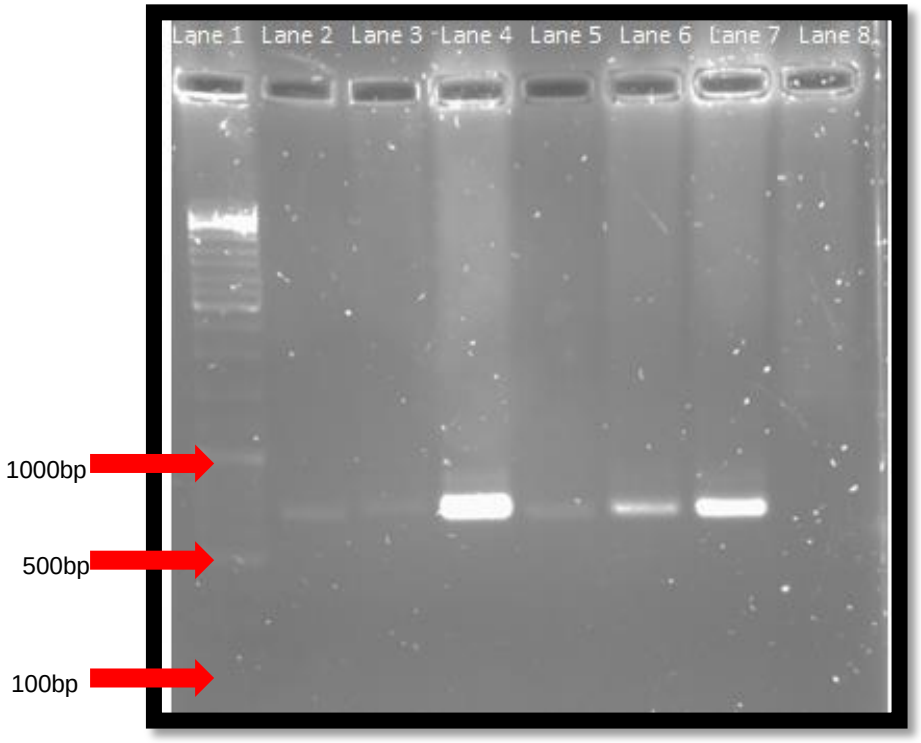

Figure 2.3.9: Gel electrophoresis results of the PCR products obtained from amplification of DNA extracted from the head and leg of an *Aedes aegypti* mosquito. Lane 1: O'GeneRuler DNA ladder mix consisting of DNA fragments between 100 – 10 000 bp (ThermoFischer Scientific, Massachusetts, USA) molecular weight marker; Lane 2: PCR product from 1:100 dilution of DNA extracted from the mosquito head; Lane 3: PCR product from 1:10 dilution of DNA extracted from the mosquito head; Lane 4: PCR product from undiluted DNA extracted from mosquito head; Lane 5: 1:100 dilution of amplified DNA extracted from a single mosquito leg; Lane 6: 1:10 dilution of DNA extracted from a single mosquito leg; Lane 7: PCR product from undiluted DNA extracted from a single mosquito leg; Lane 8: Negative control.

The extracted DNA was amplified using the HCO2198 and LCO1490 primers. The amplicon was purified and the nucleotide sequence determined using bidirectional sequencing and HCO2198 and LCO1490 primers. The nucleotide sequences were edited using Chromas Pro version 1.6, aligned using Clustal Omega version 1.2.1 and sequence data retrieved from GenBank and a BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was performed. The edited sequence was 683bp in length. A BLAST analysis of the sequence data showed 99% identity to *Aedes aegypti* (Accession number: AY432106.1), hence confirming on a molecular level that the mosquito was an *Aedes aegypti* specimen. The nucleotide sequence for this mosquito can be found in Appendix 5.3.

Representative samples for six species identified in the collections were selected. These were sixteen *Anopheles squamosus* specimens, one *Aedes aegypti*, four *Culex theileri*, one *Mansonia uniformis*, two *Ochlerotatus harrisoni* and one *Ochlerotatus juppi*. For each mosquito DNA was extracted from a single leg previously stored at -

80°C. A PCR was performed using the HCO2198 and LCO1490 primer set. The DNA amplicons of the predicted size were excised and purified, and the nucleotide sequence of the amplicons determined using Sanger sequencing. The sequence data was aligned using Clustal Omega version 1.2.1 and sequence data were retrieved from GenBank and a BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was performed. However, sequence data for *Culex theileri*, *Mansonia uniformis, Ochlerotatus harrisoni* and *Ochlerotatus juppi* mosquitoes could not be reliably edited. It is likely that these amplicons should be cloned and the sequence determined using primer sites located on the plasmid, hence the focus was on the confirmation of the genetic identification of the species *Anopheles squamosus*.

VBDF 14-04 was collected in the Free State National Botanical Gardens on the 2nd of April 2017 in a Shannon trap. The specimen was morphologically identified as *Aedes aegypti* and the molecular results are given in Table 2.3.2.

Table 2.3.2: The percentage identity of an *Aedes aegypti* mosquito obtained through BLAST analysis.

Mosquito identity number	Morphological Identification	Number of nucleotides	% identity – Blast analysis	Highest % identity obtained through BLAST
VBDF* 14-04	*Aedes aegypti*	603 bp	99%	*Aedes aegypti* (KX 420429.1)

* VBDF stands for Vector-borne Diseases Fieldwork number.

Representative specimens of *Anopheles squamosus* were selected for barcoding. Eleven specimens were collected at the Bloemfontein Zoo on the 17th of March 2017 (10-01 to 10-07, 10-09 to 10-12). Five specimens were collected from the FSNBG on the 18th of March 2017 (11-22 to 11-26). The percentage identity for all the specimens was determined following BLAST analysis (Table 2.3.3). This identity is an indication that there are some nucleotide differences in the targeted regions between members of the same species. A mean interspecific variation between 2 -5,6% is noted for most mosquito species. For *Culex* mosquitoes the average intraspecies genetic distance is 0,2%-1,4% and the inter species distance is between 7%-11,2% (Weeraratne *et al.,* 2018). *Anopheles* mosquitoes have a genetic interspecies divergence of lower than 2% (Laurito *et al.,* 2013).

Table 2.3.3: The highest percentage identity of *Anopheles squamosus* mosquitoes obtained through BLAST analysis.

Mosquito identity number	Morphological Identification	Number of nucleotides	% identity with *Anopheles squamosus* based on – Blast analysis
VBDF 10-01	*Anopheles squamosus*	638 bp	98% 95%
VBDF 10-02	*Anopheles squamosus*	617 bp	98% 95%
VBDF 10-03	*Anopheles squamosus*	612 bp	98% 95%
VBDF 10-04	*Anopheles squamosus*	602 bp	98% 95%
VBDF 10-05	*Anopheles squamosus*	602 bp	98% 95%
VBDF 10-06	*Anopheles squamosus*	602 bp	98% 95%
VBDF 10-07	*Anopheles squamosus*	602 bp	98% 95%
VBDF 10-09	*Anopheles squamosus*	551 bp	98% 95%
VBDF 10-10	*Anopheles squamosus*	571 bp	98% 95%
VBDF 10-11	*Anopheles squamosus*	601 bp	98% 95%
VBDF 10-12	*Anopheles squamosus*	616 bp	98% 95%
VBDF 11-22	*Anopheles squamosus*	565 bp	98% 95%
VBDF 11-23	*Anopheles squamosus*	624 bp	98% 95%
VBDF 11-24	*Anopheles squamosus*	527 bp	96% 95%
VBDF 11-25	*Anopheles squamosus*	581 bp	98% 95%
VBDF 11-26	*Anopheles squamosus*	618 bp	98% 95%

2.3.3. Weather data

Weather data was collected at different sampling sites in the Bloemfontein area and is given in Appendix 5.2.

Rainfall and temperature are very important for the growth and development of mosquitoes and plays a very important role in mosquito biology. The average temperatures and rainfall were obtained from the National Weather Service for the sampling period from December 2016- April 2017.

The rainfall and temperature data for the Krugersdrift Dam from December 2016 – April 2017 is shown below (Table 2.3.4). The average total rainfall for the Krugersdrift Dam area for these five months was 339mm (SD = 53,75mm). The average total rainfall for this area from 2002 – 2017 for these five months was 336,04mm (SD= 130,25mm). The average minimum temperature for the Dam for these five months was 13,24°C (SD = 3,85°C). The average minimum temperature for this area from 2002 – 2017 for these five months was 12,67°C (SD = 0,83°C). The average maximum temperature for this area for the five months was 28,84°C (SD = 3°C). The average maximum temperature for this area from 2002-2017 was 28,62°C (SD = 1,21°C). The water level of the Krugersdrift Dam was also very low over the sampling period. In March the mosquito numbers were at their highest following higher rainfall in January and February.

Table 2.3.4: Rainfall and temperature data for the sampling period of January 2017 – April 2017 at the Krugersdrift Dam.

Date	**Rainfall (mm)**	**Temperature °C**	
		Maximum	Minimum
December 2016	25,6	33,6	16,2
January 2017	120,8	28,8	15,5
February 2017	128,2	27	16
March 2017	52	29,1	11
April 2017	12,4	25,7	7,5
Total	**339**		
Average	67,8	28,84	13,24
Standard deviation	53,75	3	3,85

The rainfall and temperature data for the Free State National Botanical Gardens from December 2016 – April 2017 is shown below (Table 2.3.5). The total rainfall for the FSNBG area was 271,4mm. The average total rainfall for these five months for 2004-2017 was 322,56mm. (SD = 130,37mm) The average rainfall in 2017 was therefore above average for the area. The average minimum temperature for this site for these five months for 2004-2017 was 13,50°C (SD = 0,53°C). The maximum temperatures in the area were high with an average temperature of 30,46°C (SD =1,5°C). This is above the average temperature of 29,74°C for the site from 2004-2017. The water levels of the dam at the FSNBG were very low with isolated pools of water being present. In March the mosquito numbers were at their highest following higher rainfall in January and February.

Table 2.3.5: Rainfall and temperature data for the sampling period of December 2016 – April 2017 at the Free State National Botanical Gardens.

Date	Rainfall (mm)	Temperature °C	
		Maximum	Minimum
December 2016	42,6	34,8	15,8
January 2017	68	30,6	15,1
February 2017	116,2	28,5	16,1
March 2017	3,6	31,3	10,7
April 2017	41	27,1	7,9
Total	271,4		
Average	54,28	30,46	13,12
Standard deviation	41,54	2,94	3,64

The rainfall and temperature data for the Bloemfontein Zoo from December 2016 – April 2017 are shown below (Table 2.3.6). The total rainfall for the Bloemfontein Zoo was 413,6mm (SD = 78,01mm). The average total rainfall for the Bloemfontein Zoo from 2002-2017 for the five months is 328,92mm (SD = 122,06mm). The average minimum temperature recorded over the same period at the Bloemfontein Zoo was 14,08°C and the average maximum temperature was 29,46°C (SD = 3°C). In March the mosquito numbers were at their highest following increased rainfall occurring in January and February. The water retainers in the Bloemfontein Zoo get filled regularly and therefore they were not affected by the lower rainfall in the same way as the other areas.

Table 2.3.6: Rainfall and temperature data for the sampling period of December 2016 – April 2017 at the Bloemfontein Zoo.

Date	Rainfall (mm)	Temperature °C	
		Maximum	**Minimum**
December 2016	48,6	34	17,2
January 2017	119,6	29,5	16
February 2017	202,4	27,6	16,6
March 2017	21,8	29,7	12,6
April 2017	21,2	26	9,3
Total	413.6		
Average	82,72	29,46	14,34
Standard deviation	78,01	3	3,33

In 2016 a drought was experienced in Bloemfontein with lower than normal rainfall occurring, and rain occurred later than usual in January and February 2017. An increase in mosquito numbers was seen in the collections done in March 2017.

For the sampling period the temperatures were above average. The Bloemfontein area is showing a gradual increase in temperature with temperatures above average being recorded in the last few years. The average temperature and rainfall data for the three sampling sites and the Bloemfontein area as a whole are given in Appendix 5.2.

2.4 Summary

DNA barcoding and ecological information is used to supplement traditional morphological identification to get more conclusive results. Morphological identification of mosquitoes is the gold standard when studying mosquito populations (Chan *et al.,* 2014). Many different morphological characters are considered when looking at mosquitoes. Based on findings of research done in the Free State province, predictions can be made of the possible mosquitoes that can be expected to occur in the area.

Through morphological identification, mosquitoes from four different genera were identified: *Anopheles, Aedes, Culex* and *Mansonia*.

Species in the *Aedes* genus were found at the Bloemfontein Zoo and the FSNBG. These areas have abundant artificial containers and the FSNBG has a semi-permanent dam where these mosquitoes would be able to successfully breed. In the Bloemfontein area, *Ochlerotatus juppi* was one of the most common species found in this genus. The *Aedes* mosquitoes of southern Africa are able to overwinter and also to aestivate through dry periods (Jupp, 1996). These mosquitoes tend to be at their most active during the day, feeding mostly during the crepuscular periods (Jupp, 1996).

Culex mosquitoes are being implicated more frequently in the transmission of many diseases (Gunay *et al.,* 2015). A total of four *Culex* species were identified in this study, three in the subgenus *Culex* and one in sub-genus *Lutzia* and *Culex* were identified in the Bloemfontein area.

An *Aedes aegypti* specimen was used to validate the DNA extraction and sequencing protocols in the laboratory. *Aedes aegypti* was chosen as the control mosquito because of its distinct morphology, making morphological identification of this

specimen easy. There is a lot of sequence data available for this species as well, making barcoding very effective.

One specimen was identified as *Aedes aegypti* through morphological identification (VBDF 14-04). This mosquito was collected on the 2nd of April 2017 at the Free State National Botanical Gardens. This mosquito was positively confirmed as *Aedes aegypti* using the COI barcoding primer set.

Sixteen specimens of *Anopheles squamosus* were sequenced successfully. The sequences of these species were compared to existing sequences on GenBank. The sequences obtained in this study had a 98% identity to an *Anopheles species* that was not classified to species level and a 95% identity to *Anopheles squamosus*. These mosquito sequences have not yet been submitted to GenBank, but will be submitted in the future to aid other researchers.

Genetic data were not obtained for the *Aedes* subgenus *Ochlerotatus* species or the *Culex* species. If one barcoding region does not successfully identify a certain mosquito species, a multi-locus approach is recommended (Batovska *et al.,* 2016) (Batovska *et al.,* 2016). Alternatively, the amplicon could be cloned and the sequence determined using primer sites located on the plasmid. The sequence data could possibly be used to design more suitable primers for sequence analysis; however this was beyond the scope of the current project.

The average rainfall and temperature of the Bloemfontein area was calculated using data from 2002-2017. This data was used to determine a baseline of climactic conditions in the Bloemfontein area. Meteorological data will be collected in future and it can then be used to correlate specific weather conditions with mosquito densities. In the current study correlations between meteorological data and mosquito densities could not be determined accurately. Information about mosquito densities in the past were not available. Continued mosquito collections will be needed in the future to draw accurate comparisons between weather conditions and the mosquito densities of the area. All meteorological data can be seen in Appendix 5.2.

Temperature is one of the main factors that affects mosquito behaviour including feeding and flight behaviour and temporal and spatial distribution (Mattingly, 1969). For both the summer and winter temperatures in the Bloemfontein area it is clear to

see that above average temperatures have been experienced in the last few years. Temperature has an influence on the density of mosquitoes. Higher than usual temperatures can increase mosquito numbers and the intrinsic incubation period for mosquito-borne pathogens can be decreased significantly (Mattingly, 1969; Jupp 2005). High densities of mosquito vectors may also increase the incidence of infection of pathogens, because of a high biting rate. These factors may lead to an outbreak of disease in cases of above average temperatures (Jupp, 2005). Temperature regulates the growth and development of mosquitoes. It determines the length of each stage in the life cycle of mosquitoes and the gonotrophic cycle (Depinay *et al.,* 2004; Afrane *et al.,* 2012). Between about 18°C and 26°C a change of 1°C can alter the life span of mosquitoes by up to a week (Depinay *et al.,* 2004). Over the last six years Bloemfontein has experienced above average winter temperatures. Higher than normal winter temperatures can lead to vectors surviving for longer periods of time. This might mean that certain viruses can overwinter in vectors or vectors can survive long enough to transmit virus well into the winter (Mattingly, 1969).

Mosquitoes are very dependent on water to complete their life cycle, seeing that their juvenile stages are all dependent on water to survive. When climatic conditions like heavy rain occur, mosquito densities might increase (Jupp, 2005). Multiplication of viruses in these mosquitoes can also be increased (Jupp, 2005). Ironically, this rarely happens. Increased rainfall tends to be associated with lower temperatures, so the important relationship is more likely to be alternating periods of high rainfall and high temperatures. That way female mosquitoes are stimulated to take a blood-meal and to oviposit, leading to rapid larval development (pupal development is more or less universal, regardless of physical conditions) and rapid virus replication, followed again by rain-stimulated oviposition and blood-feeding. These factors may lead to an outbreak of disease, seeing that high mosquito densities are needed for WNV transmission (Rosa *et al.*, 2014). Severe weather conditions can also trigger outbreaks of disease. Droughts and floods can create breeding sites for mosquitoes. During drought periods water can become more concentrated in smaller areas and mosquitoes will use these water bodies as breeding sites. The eggs of some mosquitoes can withstand dry conditions. These eggs can remain viable until further rain is experienced. These eggs will then hatch, and this could lead to high mosquito densities following drought periods. Severe climatic conditions could also lead to a

decrease in the number of mosquito predators. This would lead to an increase in mosquito numbers (Osorio *et al.,* 2008). During the study period, above average temperatures were experienced, with lower than usual rainfall. This could influence mosquito densities in the future. In southern Africa it was shown that diseases caused by alphaviruses in horses occur mostly during March and April (van Niekerk *et al.,* 2015). This could be attributed to the higher density of mosquitoes over this period. This is consistent with results obtained in this study, where the highest density of mosquitoes was recorded in March.

Chapter 3: Screening of wild caught mosquitoes in the Bloemfontein area for the evidence of arboviruses.

3.1 Introduction

Annually, nearly a billion people are affected by vector-borne diseases globally, with nearly 1.3 million deaths (Werblow *et al.,* 2016). Outbreaks of arboviral disease occur annually in South Africa usually associated with rainfall favouring mosquito breeding. The extent of the outbreaks varies, and it is likely that during small outbreaks disease may be unrecognised. Many arboviral diseases have similar presentation to common infections and in the instances where disease is mild, it can be misdiagnosed without laboratory confirmation. Surveillance of mosquito populations contributes to identifying which viruses are circulating within specified areas.

In South Africa arboviruses such as SINV, WNV, WSLV and RVFV are the arboviruses most frequently associated with disease. WNV and SINV have a similar ecology in South Africa (Jupp, 2005) with *Culex* mosquitoes playing a role as the main vectors. Birds, especially water birds act as the main reservoirs of WNV and SINV (Rautenbach, 2011).

SINV was first isolated in South Africa in 1954 from a single pool made up of a mixture of *Culex pipiens/quinquefasciatus, Culex univittatus* and *Culex theileri*, all from Springs in late summer/ early autumn (Weinbren *et al.* 1956). In 1963 SINV was isolated from skin lesions from a patient that showed signs of SINV infection. In 1974 an outbreak occurred in the Northern Cape Province and the Karoo region (Storm, 2014). SINV is mostly isolated in the Gauteng, Northern Cape and Free State provinces of South Africa (Storm, 2014; Venter, 2018). The outbreak in 1974 coincided with an outbreak of WNV. Similarly, in 1983/84 there was an outbreak of both SINV and WNV in the Gauteng province (Burt *et al*., 2014). SINV produces irregular epidemics in South Africa with many of the infections seen in the Highveld area. Summer rainfall in this area plays a critical role, but this is also the preferred habitat of the main vector, *Culex univittatus* and the secondary vector, *Culex theileri. Culex univittatus* feeds on birds that are most likely not exposed to previous arboviral infections and are therefore susceptible. This includes newly-hatched passerine (Passeridae) birds, especially the

house sparrow (*Passer domesticus*), an influx of antibody-free migrating swallows (*Hirundo rustica*) and water birds and population explosions of quelea (*Quelea* spp.). *Culex theileri*, also makes use of this increased food source and acts as bridging vectors to horses and humans. SINV is associated with fever, rash and arthralgia in humans (Venter, 2018).

The flavivirus, WNV, has a widespread distribution, being enzootic in Africa, Asia, Europe and Australia (Campbell *et al.,* 2002; Turell *et al.*, 2001). In 1973/1974 there was a large outbreak of WN fever in South Africa, possibly the largest ever recorded (McIntosh *et al.* 1976; Campbell *et al*., 2002, Rautenbach, 2011). Human infections are most commonly identified in the moister regions of the Highveld. Both WNV and SINV are endemic in the Highveld and Karoo regions (Uejio, *et al.,* 2012; Venter, 2018).

Several species of *Culex* mosquitoes are responsible for transmission of WNV and SINV (Mattingly, 1969; Turell *et al.*, 2001). More than 40 mosquito species have been identified as carriers of WNV in Africa, Europe and Asia. *Culex univittatus* acts as the primary mosquito vector in the Karoo region, while *Culex neavei* is the primary vector in KwaZulu Natal. These mosquitoes lay their eggs in ground water, because they cannot survive any form of desiccation. *Culex univittatus* prefers to populate areas with emergent vegetation and they also require clear, clean water (Jupp, 2005). *Culex univittatus* mosquitoes also have a very low human biting rate as they are primarily ornithophilic (Jupp, 2005). WNV can overwinter in mosquito vectors and then amplify during the spring time (Turell *et al.*, 2001).

There is a maintenance transmission cycle between these mosquitoes and wild birds. Birds act as the amplifying hosts for the virus (Goddard *et al.*, 2002; Jupp, 2005; Rautenbach, 2011; Rosa *et al.*, 2014). Humans are poorly viraemic and therefore act as dead-end hosts in the transmission cycle (Jupp, 2005; Rosa *et al.*, 2014).

Wesselsbron disease is an acute infection of sheep and cattle in Africa (Weyer *et al.* 2013; Diagne *et al.* 2017). This disease is caused by WSLV. Wesselsbron disease was first isolated in 1955 in Wesselsbron, Free State province, South Africa. This isolation was made from a febrile man and a dead lamb during an outbreak (Weyer *et al.* 2013; Diagne *et al.* 2017). Apart from South Africa, WSLV has been isolated from mosquito species in Botswana, Cameroon, Central African Republic, Democratic

Republic of Congo, Madagascar, Mauritania, Mozambique, Nigeria, Senegal, Uganda, and Zimbabwe. WSLV is mostly associated with mosquitoes in the *Aedes* genus (Diagne *et al.,* 2017).

In sheep Wesselsbron disease is similar in clinical presentation to Rift Valley fever (RVF). This disease causes abortion and mortality in pregnant ewes (20%) as well as mortality in lambs and goat kids (Weyer *et al.* 2013; Diagne *et al.* 2017; Venter, 2018). In lambs and goat kids the infection is short lived with fever, anorexia, general weakness and respiratory distress being common (Weyer *et al.* 2013). Infections have also been documented in goats, cattle and pigs. Infections in these animals cause less severe fever than is seen in sheep. Neurological disease has also been described in horses in South Africa. WSLV outbreaks regularly occur in concurrence with RVF outbreaks in South Africa and therefore tend to get overlooked (Diagne *et al.,* 2017). Although Wesselsbron disease is found mainly in sheep, cattle and goats, infections with WSLV do occur in humans. Human infections are short lived, causing very mild arthralgia, myalgia and fever (Weyer *et al.* 2013; Diagne *et al.* 2017).

Potential vector mosquitoes for SINV, WNV and WSLV have been identified in the Bloemfontein area. Surveillance of mosquitoes for the presence of viruses can be performed using virus isolation and detection of viral nucleic acid. Many arboviruses have to be handled within the confines of biosafety level 3 and 4 laboratories depending on the category of classification, which severely limits the number of laboratories that can attempt isolations. In the absence of performing viral isolation, the use of molecular methods to detect viral nucleic acid plays an important role. Molecular methods provide a safe and sensitive method for performing surveillance. However, there are no standardised assays for molecular detection. Various primer sets have been described in the literature, however for this study it was decided to use primer pairs previously designed in-house, and to determine their ability to detect control RNA for selected arboviruses and finally, to use the assays to screen the mosquitoes, that were collected in the study for morphological and genetic identification, for selected arboviruses.

3.2 Material and Methods

3.2.1 Development of a real-time RT-PCR molecular assay for flaviviruses and Sindbis virus

3.2.1.1 Primer design

Sindbis primers

A primer pair was previously designed to amplify the NSP2 region of the Sindbis SAAR86 strain in an unrelated study (Hanekom, 2014) (Table 3.2.1).

Table 3.2.1: Oligonucleotide primers for amplification of SINV RNA.

Name of Primer	5' → 3' sequence	Genomic position	Size of product
Sindbis NSP2 forward primer	5'-GAC AGT ATA TCG TTG TCT CG CC -3'	1744 -1766	182 bp
Sindbis NSP2 reverse primer	5'- GAA TTC TGG CCA TGG TAC GGC -3'	1926-1905	182 bp

Flavivirus primers

A consensus primer pair targeting the NS5 regions of flaviviruses was designed in a previous study (Samudzi, 2008). The primers were designated FlaviF1 and FlaviR1 and target a ~414 bp region of the NS5 gene (Table 3.2.2).

Table 3.2.2: Primer sequence and genomic position of a 414bp region of the flavivirus NS5 gene for the use of flavivirus identification.

Name of Primer	5' → 3' sequence	Genomic Position	Size of product
Flavi F1	5'-ATG GCH ATH ACW HAC AC-3'	8847-8863	414 bp
Flavi R2	5'- CCA CAT GWA CCA DAT GGC -3'	9259-9242	414 bp

The primers were used in RT-PCR reactions. SINV RNA was reverse transcribed using Superscript™III reverse transcriptase according to manufacturer's instructions. The template and primer mix was prepared as follows: 1 µl of the 2 pmol/µl Sindbis NSP2 forward primer, 1 µl of 10 mM PCR deoxynucleotide mix, 1 µl RNA template and 10 µl of nuclease free water to a final reaction volume of 13 µl. Negative controls were set up exactly as described with the 1 µl of RNA template being substituted with 1 µl of NFW.

The master mix for cDNA synthesis was prepared as follows: 4 µl of 5X first strand buffer, 1 µl of 0.1M DTT, 0.5µl RNaseOut, 1 µl of 200 U/µl Superscript™RT enzyme and 0.5 µl of NFW to a final reaction volume of 7 µl.

First strand cDNA was synthesized using the following reaction conditions: The primer and template mix were incubated in a Proflex PCR System thermocycler (Life Technologies, Carlsbad, California, USA) at 65^0C for five minutes and 4°C for one minute. Subsequently, 7 µl of master mix was added to the primer and template mix. The reaction mix was held at 4^0C for one minute, 50^0C for 60 minutes, 85^0C for five minutes and lastly samples were held at 4^0C indefinitely. The first strand cDNA was used as the template for downstream reactions.

The cDNA template was amplified as follows: 10 µl 5x green GoTaq flexi buffer, 4 µl 25 mM $MgCl_2$ solution, 1 µl 10 mM PCR nucleotide mix, 1µl 20pmol/µl Sindbis NSP2 forward primer, 1 µl 20 pmol/µl Sindbis NSP2 reverse primer, 0.25 µl 5U/µl GoTaq®G2 hot start polymerase, 1 µl cDNA template and 31.75 µl NFW to a final reaction volume of 50 µl. The reaction was cycled at 95^0C for two minutes followed by 35 cycles of 95^0C for 30 seconds, 50^0C for 30 seconds, 72^0C for one minute. The sample was incubated at 72^0C and held at 4^0C indefinitely.

A 10 µl aliquot of the PCR product was separated using a 1% agarose gel prepared in TAE buffer (pH 8.5) containing 4000x GelRed® (Biotium, Fremont, California, USA). O'GeneRuler DNA ladder mix comprising DNA fragments from 100 to 10 000 bp fragments (ThermoFischer Scientific, Massachusetts, USA) was used to determine the size of the amplicons. The samples were loaded in a 2000x loading dye solution containing GelRed® (Biotium, Fremont, California, USA). Gel electrophoresis was performed using a BioRad PowerPac Basic system at 80V for 60 minutes. The DNA bands were visualized with a UV transilluminator.

3.2.1.2. Preparation of positive controls for molecular assay

WNV and WSLV require BSL 3 facilities for culture hence transcribed RNA was prepared for the use as positive controls. Transcribed RNA was prepared from a region of the NS5 gene of each virus that was amplified from RNA (kindly supplied by Professor Paweska, National Institute for Communicable Diseases (NICD) in

Johannesburg, South Africa) for a previous study and stored at -80°C. The WNV RNA was isolated from SA93/01 strain and WSLV from SAH-177 strain. The amplified regions were cloned for transcription.

SINV can be cultured within a biosafety level 2 facility, hence SINV RNA used in this study was extracted from infected cell culture supernatant obtained from a previous study and stored at -80°C.

Preparation of plasmids for transcription of flavivirus RNA

WNV and WSLV RNA were reverse transcribed using Superscript™III reverse transcriptase (Life Technologies, Carlsbad, California, USA) according to manufacturer's instructions. The template and primer mix was prepared as follows: 1 µl of the 2 pmol/µl FlaviF1 primer, 1 µl of 10 mM PCR deoxynucleotide mix, 1 µl RNA template and 10 µl of nuclease free water to a final reaction volume of 13 µl. Negative controls were set up exactly as described with the 1 µl of RNA template being substituted with 1 µl of NFW.

The master mix for cDNA synthesis was prepared as follows: 4 µl of 5X first strand buffer, 1 µl of 0.1 M DTT, 0.5µl RNaseOut™(Life Technologies, Carlsbad, California, USA), 1 µl of 200 U/µl Superscript™RT enzyme (Life Technologies, Carlsbad, California, USA) and 0.5 µl of NFW to a final reaction volume of 7 µl.

First strand cDNA was synthesized using the following reaction conditions: The primer and template mix were incubated in a Proflex PCR System thermocycler (Life Technologies, Carlsbad, California, USA) at 65^0C for five minutes and 4°C for one minute. Subsequently, 7 µl of master mix was added to the primer and template mix. The reaction mix was held at 4^0C for one minute, 50^0C for 60 minutes, 85^0C for five minutes and lastly samples were held at 4^0C indefinitely. The first strand cDNA was used as the template for downstream reactions.

The cDNA template was amplified as follows: 10 µl 5x green GoTaq® flexi buffer (Promega, Madison, Wisconsin, USA), 4 µl 25 mM $MgCl_2$ solution, 1 µl 10 mM PCR nucleotide mix, 1 µl 20 pmol/µl type specific forward primer, 1 µl 20 pmol/µl type specific reverse primer, 0.25 µl 5U/µl GoTaq®G2 hot start polymerase (Promega,

Madison, Wisconsin, USA), 1 µl cDNA template and 31.75 µl NFW to a final reaction volume of 50 µl. The reaction was cycled at 95^0C for two minutes followed by 35 cycles of 95^0C for 30 seconds, 50^0C for 30 seconds, 72^0C for one minute. The sample was incubated at 72^0C and held at 4^0C indefinitely.

A 10 µl aliquot of the PCR product was separated using a 1% agarose gel prepared in TAE buffer (pH 8.5) containing 4 000x GelRed® (Biotium, Fremont, California, USA). O'GeneRuler DNA ladder mix comprising DNA fragments from 100 to 10 000 bp fragments (ThermoFischer Scientific, Massachusetts, USA) was used to determine the size of the amplicons. The samples were loaded in a 2000x loading dye solution containing GelRed® (Biotium, Fremont, California, USA). Gel electrophoresis was performed using a BioRad PowerPac Basic system at 80V for 60 minutes. The DNA bands were visualized with a UV transilluminator. The agarose gels were used to determine if the primer pair can effectively amplify WSLV and WNV DNA and that the primers function correctly under the laboratory settings used in this study.

The bands of interest were excised. The excised DNA bands were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, Wisconsin, USA), according to manufacturer's instructions. The DNA was eluted in 30 µl of NFW by centrifugation at 16 000xg for one minute. The DNA was quantified using a Nanodrop 2000 spectrophotometer (Life Technologies, Carlsbad, California, USA). The purity was determined from the 260 nm:280 nm ratio of absorbance and stored at -20^0C until being used in downstream reactions. The WNV and WSLV amplicons were used to prepare constructs for the transcription of viral RNA.

Cloning of partial NS5 gene into pGEM® T Easy bacterial vector using TA cloning

The high-copy number pGEM® T Easy Vector contains T7 and SP6 RNA polymerase promoters flanking multiple cloning regions within the α-peptide coding region of the enzyme ß-galactosidase. The vector map and multiple cloning sites of the pGEM®-T easy vector are shown in Figure 3.2.1.

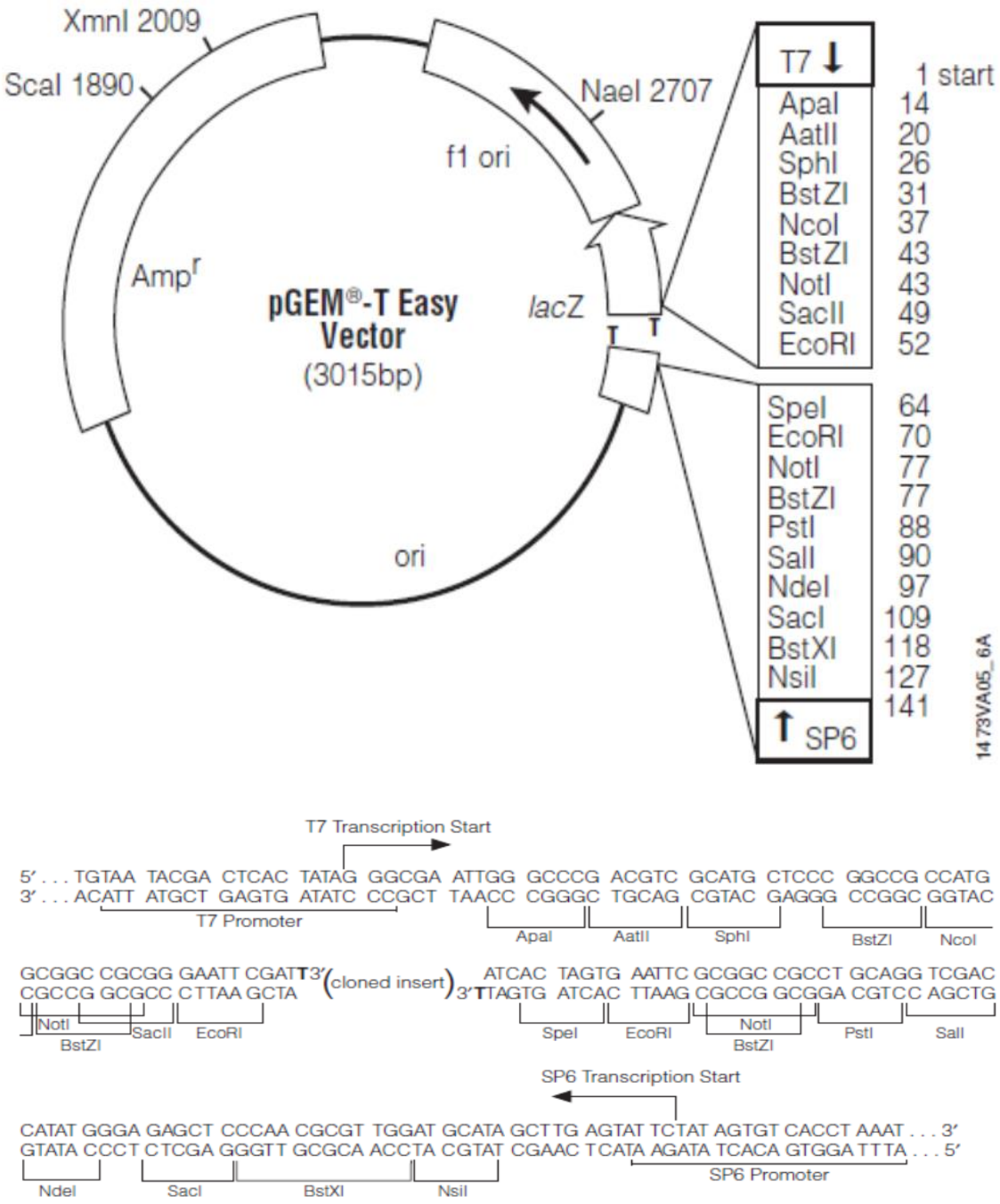

Figure 3.2.1: Vector map and sequence reference points of pGEM®-T easy vector (Promega, Madison, Wisconsin, USA)

The WNV and WSLV amplicons were ligated into pGEM®-T easy vector (Promega, Madison, Wisconsin, USA) by TA cloning using T4 DNA ligase and using chemically competent JM109 E. coli host cells (Promega, Madison, Wisconsin, USA). These cells have a transformation efficiency of 1 x 10^8 cell forming units/µg.

The pGEM®-T easy vector is a vector that is linearized at base 60 with a single 3'-

terminal thymidine at the 3' end. This vector also has a high copy number and contains T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the alpha peptide coding region of the enzyme beta galactosidase. The pGEM®-T Easy vector contains the *lacZ* gene that encodes β-galactosidase. This enzyme is responsible for breaking down galactose into its breaking blocks lactose and glucose. The enzyme can convert X-gal (5-bromo-4-chloro-3-indolyl-[beta]-D-galactopyranoside), a product that is normally colourless, to a blue product. Within the *lacZ* gene there are multiple cloning sites where DNA fragments can be inserted. If the DNA fragment is inserted correctly the *lacZ* gene will be interrupted. This will lead to the β-galactosidase enzyme being non-functional and therefore no blue product will be formed. This will mean that all positive transformed bacterial colonies will appear blue in colour and all non-transformed colonies will have a normal white appearance. DNA was transformed using manufacturer's instructions.

The WNV and WSLV partial NS5 gene was ligated into pGEM® T Easy vector generating pGEM-NS5 constructs. Ligation reactions were prepared as shown in Table 3.2.3.

Table 3.2.3: Ligation reaction components for the pGEM-NS5 construct.

Reaction components	**Insert (NS5 gene)**	**Positive control**	**Background control**
2x Rapid ligation buffer, T4 DNA ligase	5 µl	5 µl	5 µl
pGEM T easy vector (50ng)	1 µl	1 µl	1 µl
PCR amplicon (Insert)	0,5 µl (1-3 ng)	-	-
Control insert DNA	-	2 µl	-
T4 DNA ligase (3 Weiss units/µl)	1 µl	1 µl	1 µl
Nuclease free water	-	1 µl	3 µl
Total	**10 µl**	**10 µl**	**10 µl**

To determine how much of the PCR product was required the following calculation was used:

$$\left(\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of the vector}}\right) \times \text{the ratio of vector to vector molar ratio} = \text{ng of the insert}$$

The following was used in calculating the amount of insert needed in the ligation reaction:

A total of 50 ng of vector, 0,414 kb of insert, 3 kb of vector and a vector to vector molar ratio of 1:3.

A volume of 0,5µl of each of the two amplicons were added to their respective ligation reactions. Ligation reactions were incubated at 4^0C overnight to ensure the maximum transformation of cells. A positive control and background control were included for both WNV and WSLV. The positive control only contains control plasmid DNA and no PCR amplicon. The background control contains all reagents but excluding the control plasmid DNA and the PCR amplicon.

Transformation of chemically competent JM 109 cells

A 50 µl aliquot of JM 109 cells (Promega, Madison, Wisconsin, USA) was used for each transformation reaction. Transformation of the cells using the plasmid was performed using the heat shock method. Competent cells have modified membranes that facilitate the uptake of plasmid when subjected to heat shock. A volume of 2 µl of ligation reaction mix (Table 3.1) was added to the 50 µl of competent cells and incubated on ice for 20 minutes. Cells were heat-shocked at exactly 42^0C for 50 seconds and immediately put on ice for two minutes. A volume of 950 µl super optimal broth with catabolite repression (SOC) media was added to the ligation reaction mix. The ligation/transformation reaction was incubated for 1.5 hours at 37^0C shaking at approximately 150rpm.

A total volume of 100µl of each transformation culture was plated in duplicate on Luria Bertani (LB) plates containing ampicillin (amp) at a final concentration of 100µg/ml, isopropyl β-D-thionalactopyranoside (IPTG) (ThermoFischer Scientific, Massachusetts, USA) and 5-bromo-4-chloro-3-indoyl-β-D-galactopyranosidase (X-gal) (ThermoFischer Scientific, Massachusetts, USA).

The LB plates were prepared as follows: 10 g Bacto-tryptone, 5 g-Bacto yeast, 10 g sodium chloride, 15 g agar and distilled water to a final volume of one litre. The broth was autoclaved at 121ºC for approximately 30 minutes and allowed to cool to 50ºC before adding ampicillin.

A final volume of approximately 30-35 ml of broth was added to 85 mm petri dishes and allowed to solidify. The X-Gal/IPTG plates were prepared by applying a volume of 40µl of X-gal stock solution at a final concentration of 20 mg/ml and 4 µl volume of a 200 mg/ml of IPTG. X-gal and IPTG were spread over the entire surface of the plate and incubated at 37^0C until the fluid was no longer visible.

A 300 µl aliquot of cells from the ligation-transformation mix was spread over the plate which was incubated overnight at 37ºC.

Confirmation of positive transformants

Blue/white colony selection was used for screening of transformants. The beta galactosidase converts the colourless substrate X-gal to produce blue colonies. *The lacZ* gene contains the multiple cloning and A/T cloning sites. The gene will be disrupted in positive transformants, therefore β-galactosidase will no longer be produced and X-gal can no longer be metabolised to produce blue colonies. Colonies containing positive transformants will thus be white. If the insert does not disrupt the *lacZ* gene, positive transformants may appear blue. Therefore, it is necessary to confirm positive transformants.

In order to, identify positive transformants three white colonies were selected from the LB/ampicillin/IPTG/X-gal plates of each ligation reaction and the colonies were designated WNV 1, WNV 2, WNV 3, WSLV 1, WSLV 2 and WSLV 3. These colonies were grown overnight in a 5 ml volume of LB/amp at 37ºC with shaking at 200rpm.

After sixteen hours of incubation plasmid DNA was purified using the centrifugation protocol of the PureYield™Plasmid Miniprep system (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions. Briefly 1.5 ml bacterial culture was transferred into a microcentrifuge tube and centrifuged at 14 000xg for 30 seconds; this was done twice to obtain a high yield of purified plasmid DNA. A volume of 600 µl TE buffer (pH 8.0) was added to the cell pellet and resuspended completely. A 100 µl aliquot of cell lysis buffer and 350 µl neutralization solution were added to the bacterial culture. The reaction mixture was inverted six times and centrifuged at 14

000xg for three minutes. The supernatant was transferred to a Pure Yield™ Mini-column and centrifuged at 14 000xg for 15 seconds. Wash steps using endotoxin removal and column wash solution were performed to get rid of residual debris. Lastly plasmid DNA was eluted in 30 µl elution buffer. The DNA concentration of the plasmid DNA was measured by using a Nanodrop 2000 spectrophotometer. The eluted plasmid DNA was stored at -20^{0}C. Conformation and correct orientation of positive transformants were confirmed by restriction digestion and plasmid DNA PCR respectively. Glycerol stocks of each pGEM overnight culture was prepared. 850 µl pGEM overnight culture was added to 150 µl glycerol and stored in cryotubes (Nunc, Denmark) at -70ºC.

Restriction enzyme digests

To verify that the plasmid DNA was successfully ligated into pGEM®-T easy vector, each clone was analysed by restriction enzyme digestion with restriction enzymes for excision of the WNV and WSLV NS5 gene. The vector was linearized using the restriction enzyme, *Not1* which recognizes two sites located within the multiple cloning site of the pGEM®-T easy vector at positions 43 and 491. The restriction enzyme digestion reaction components are shown in Table 3.2.4.

Table 3.2.4: Reaction components of the restriction digestion using *Not1* restriction enzyme

Reaction components	**Volume**
Not 1 (10 U/µl)	1 µl
Plasmid	1 µl
10x restriction enzyme, Buffer D	2 µl
Nuclease free water	16 µl
Total	**20 µl**

The restriction digestion reaction was incubated at 37^{0}C for two hours, the products were separated by electrophoresis on 1% agarose gel at 80V for 60 minutes and visualised under a UV transilluminator.

A positive transformant for WNV and for WSLV were selected and the partial NS5 region was amplified using SP6 reverse primer which targets a site present on the pGEM®-T easy vector and the FlaviF1 forward primer. This reaction was performed to obtain template for transcription of RNA and to confirm the correct orientation of the

gene of interest. The primers that flank the multiple cloning sites of the pGEM®-T easy vector are shown in Table 3.2.5.

Table 3.2.5: Primers that flank the multiple cloning site of the pGEM®-T easy vector

Primer name	Forward/reverse	Nucleotide sequence	*T_m	Length
SP6 primer	Reverse	5'ATTTAGGTGACACTATAG3'	50^0C	18 bp

*T_m and GC content calculated using Promega biomath. (www.**promega**.com/a/apps/**biomath**/?calc=tm).

Amplification of the plasmid DNA was performed as follows: 10 µl of 5x Green GoTaq®flexi buffer (Promega, Madison, Wisconsin, USA), 4 µl of a 25 mM $MgCl_2$, 1µl of PCR nucleotide mix (10 mM for each dNTP), 1 µl of 20 pmol/µl FlaviF1 forward primer, 1 µl of 20 pmol/µl SP6 reverse primer, 0.25 µl of 5 U/µl GoTaq®G2 Hot Start Polymerase (Promega, Madison, Wisconsin, USA), 1 µl of DNA plasmid and 31.75 µl nuclease free water to a final reaction volume of 50 µl.

The reaction was cycled using the following cycling conditions: initial denaturation for one minute at 95^0C followed by 25 cycles of denaturation at 95^0C for one minute, annealing temperature at 50^0C for one minute, extension 72^0C for one minute and extension at 72^0C for five minutes and samples were held at 4^0C indefinitely.

The PCR amplicons were visualised on a 1% agarose gel and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, Wisconsin, USA), according to manufacturer's instructions. The DNA was eluted in 30 µl of NFW by centrifugation at 16 000xg for one minute. The DNA was quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Illinois, USA). The purity was determined from the 260 nm:280 nm ratio of absorbance and stored at -20^0C until being used in downstream reactions.

DNA sequencing of WNV and WSLV NS5 gene in pGEM® T Easy vector

The nucleotide sequence of each amplicon was determined using the FlaviF1 forward primer (0.8 pmol/µl) and the SP6 reverse primer (0.8 pmol/µl) using the BigDye® Terminator V3.1 sequencing ready reaction kit as previously mentioned (Section 2.2.3.4). The sequence reaction was purified using EDTA/ethanol precipitation. Samples were stored at 4^0C until submission to the Department of Microbial,

Biochemical and Food Biotechnology, University of the Free State, Bloemfontein in SA. To confirm each positive control, nucleotide sequences were edited using Chromas Pro version 1.6, aligned using Clustal Omega version 1.2.1 and sequence data retrieved from GenBank and a BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was performed.

RNA transcript

RNA was transcribed from the plasmid DNA template using SP6 MEGAscript®Kit (Ambion Inc., Texas, USA) according to manufacturer's instructions. Briefly, an RNA transcription reaction component mix was made up as follows for each dilution: 1 µl 5 mM ATP, 1 µl 5 mM GTP, 1 µl 5 mM CTP, 1 µl 5 mM UTP, 2 µl 10x reaction buffer, 2 µl 20 U/µl SP6 enzyme mix, 1-3 ng plasmid DNA and nuclease free water up to a final reaction volume of 20 µl.The reaction mixture was incubated at 37^0C for 16 hours.

The RNA transcripts were purified using the SV Total RNA Isolation System (Promega, Madison, Wisconsin, USA) according to manufacturer's instructions. Briefly the 20 µl transcript reaction was transferred into a micro-centrifuge tube containing 175 µl RNA lysis buffer. A volume of 350 µl of dilution buffer was added to the lysate and mixed thoroughly by inverting four times and the lysate was incubated at 70^0C for three minutes. The lysate was centrifuged for 10 minutes at 14 000 x g. A 200 µl aliquot of 95% ethanol was added to the cleared lysate, mixed and centrifuged at 14 000 x g for one minute through a spin column. The column was washed with 600 µl RNA wash solution. DNA was removed by incubation with 50 µl of DNase incubation mix (40 µl yellow core buffer, 5 µl 0.09 M $MnCl_2$ and 5 µl of DNase 1 enzyme) at 25^0C for 25 minutes. The enzyme activity was stopped by using DNase stop solution and the spin column washed to remove potential inhibitors. The RNA was eluted in 30 µl nuclease free water and stored at -80^0C for downstream application. The plasmid derived RNA was used as the positive control for downstream qPCR reactions. To ensure that no DNA remained after the DNase treatment a PCR reaction was performed without the reverse transcription step using RNA diluted 1:100 and 1:1000 as template and the products separated and visualized on a 1% agarose gel.

To confirm that transcribed RNA for WNV and WSLV were suitable as controls, a two-step RT-PCR was performed. The RNA was reverse transcribed using Superscript™III

reverse transcriptase. The resulting cDNA was amplified using GoTaq®G2 hot start polymerase. PCR products were separated on a 1% agarose gel. The PCR products were excised from the agarose gel and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, Wisconsin, USA). Purified DNA made from the RNA controls was quantitated using the Quant-iT™ dsDNA BR Assay kit with the Qubit fluorometer (Life Technologies, Carlsbad, USA) according to manufacturer's instructions. Briefly, a Quant-iT™ dsDNA BR working solution was made by diluting the Quant-iT™ dsDNA BR reagent 1:200 in Quant-iT™ buffer. Two standard assay reactions were set up as follows: Standard one was made up of 190 µl of working solution and 10 µl of the 0 ng/µl standard to a total volume of 200 µl.Standard two was made up of 190 µl of working solution and 10 µl of the 10 ng/µl standard to a total volume of 200 µl. The SINV sample was prepared as follows: 199 µl of working solution and 1 µl of RT-PCR amplicon to a total volume of 200 µl. The DNA concentrations were used to calculate the copy numbers of DNA. This was done using the DNA concentration to copy number calculator from URI Genomics and Sequencing Centre (cels.uri.edu/gsc/cndna.html).

To confirm that there was no DNA contamination of the transcribed RNA controls, transcribed RNA was tested using PCR RNA diluted 1:100 and 1:1 000.

3.2.2. Development of realtime RT-qPCR.

Primer and probe design

Flaviviruses: The FlaviF1 forward primer and FlaviR1 reverse primer were used. A Flavivirus probe (FLAVI+LNA) was designed based on the inner region of the NS5 gene that is flanked by FlaviF1 and FlaviR1 binding regions. The flavivirus probe was designed in a previous study (Mathengtheng, 2015). The probe was labeled with a 6-carboxyfluorescein (6FAM) dye on the 5' end and coupled to an IOWA Black™ FQ quencher (IABkFQ) on the 3'end. Due to the limitations in length being consequential to low melting temperatures and stability, the probe designated Flavi+LNA was modified to include locked nucleic acids (LNAs) which are made of nucleic acid analogues in which the ribose ring is locked by a methylene bridge connecting the 2'-O atom and the 4'-C atom, making it inaccessible for base-pairing. This increases the

melting temperature and lends more stability to the probe. The probe information for the Flavi+LNA probe is shown in Table 3.2.6.

Alphaviruses: a Sindbis virus specific probe was designed in a previous study (Hanekom, 2014). This is a TaqMan hybridization probe designated Sindbis NSP2 TaqMan probe. The TaqMan reaction requires a hybridization probe that is labelled with two different fluorescent dyes. One of these dyes acts as a reporter dye. In the case of the Sindbis NSP2 TaqMan probe the 5' end of the probe was labelled with a 6-FAM (6-carboxyfluorescein) fluorophore. This fluorophore has an emission wavelength 0f 520 nm. The other dye is known as a quenching dye. In the case of the Sindbis NSP2 TaqMan probe the 3' end of the probe was labelled with IABkFQ. This is an IOWA Black™ FQ quencher (Heid *et al.* 1996; Brookman-Amissah et al. 2017). Quenchers are similar to the fluorophores, but rather than emitting the fluorescence energy they absorb as light, they can turn it into heat. This means that the probe will not produce fluorescence as long as the quencher remains in place (Brookman-Amissah *et al.* 2017).

During the extension phase of the PCR reaction, the hybridization probe will be cleaved off of the template molecule by the 5'-3' nucleolytic activity of the DNA polymerase. When the probe is cleaved the fluorophore is no longer quenched and the fluorescence increases (Brookman-Amissah *et al*. 2017). This change in fluorescence was measured by the LightCycler 2.0 (Roche, Manheim, Germany).

The Sindbis NSP2 TaqMan probe contains an internal ZEN quencher. These quenchers are used in double-quenching probes to provide an increased signal with less background signal noise. Where traditional probes normally have a distance of 20-30 bases between the quencher and the reporter, double-quenched probes only have a distance of about 9 bases between the reporter and the first quencher. This increases precision and allows the amplification of much longer DNA templates (Brookman-Amissah et al. 2017). The probe information for the Sindbis NSP2 TaqMan probe is shown in Table 3.2.6.

Table 3.2.6: Nucleotide sequence of probes specific to a region of the NSP2 gene of SINV and a region of the NS5 gene of flaviviruses.

Probe	
Name of probe	5' → 3' sequence
Flavi+LNA	(5'-/56-FAM/ TAC AWC ATG A+T+G +GG /3IABkFQ/ -3'
Sindbis NSP2 TaqMan probe	(5'-/56-FAM/ATC ATA ACG /ZEN/ CAC TCC GGA AGA TCA GGA /3IABkFQ/ -3'

Flavivirus two step RT-qPCR

The flavivirus two step RT-qPCR was performed using the LightCycler® TaqMan® Master kit (Roche, Manheim, Germany) according to parameters recommended by the supplier. In brief, the reaction setup consisted of 2 µl of 10 pmol/µl FlaviF1 forward primer, 2 µl of 10 pmol/µl FlaviR1 reverse primer, 2 µl of 2 pmol/µl Flavi+LNA hydrolysis probe, 4 µl of the LightCycler® TaqMan® 5X master mix (Roche, Manheim, Germany), 1 µl of DNA template and nuclease free water to a total reaction volume of 20 µl. A total of 1 µl of DNA template was used for positive controls. The RT-qPCR assay was performed in a LightCyler® 2.0 instrument (Roche, Manheim, Germany) which is a carousel-based system. For the negative controls the 1 µl of DNA template was replaced with 1 µl of nuclease free water. The cycling conditions for the flavivirus RT-qPCR can be seen in Table 3.2.7.

Table 3.2.7: qPCR cycling conditions for the flavivirus TaqMan® RT-qPCR assay.

Programme name	**Analysis mode**	**Cycles**	**Target temperature**	**Hold time**	**Acquisition**
Pre-Incubation					
	None	1	95°C	10 minutes	None
Amplification					
Denaturation	Quantification	45	95°C	10 seconds	None
Annealing			55°C	40 seconds	None
Extension			72°C	1 second	Single
Cooling					
	None	1	40°C	30 seconds	None

Sindbis virus two step RT-qPCR

The SINV two step RT-qPCR was performed using the LightCycler® TaqMan® Master kit (Roche, Manheim, Germany) according to parameters recommended by the supplier. In brief, the reaction setup consisted of 2 µl of 10 pmol/µl Sindbis NSP2 forward primer, 2 µl of 10pmol/µl Sindbis NSP2 reverse primer, 1 µl of 2 pmol/µl Sindbis NSP2 TaqMan probe, 4 µl of the LightCycler® TaqMan® 5X master mix, 1 µl of DNA template and nuclease free water to a total reaction volume of 20 µl. A total of 1 µl of DNA template was used for positive controls. The PCR assay was performed in a LightCyler® 2.0 instrument (Roche, Manheim, Germany) which is a carousel-based system. For the negative controls the 1 µl of DNA template was replaced with 1 µl of nuclease free water.

The cycling conditions for the flavivirus qPCR can be seen in Table 3.2.8.

Table 3.2.8: RT-qPCR cycling conditions for the SINV TaqMan® two step RT-qPCR assay.

Programme name	**Analysis mode**	**Cycles**	**Target temperature**	**Hold time**	**Acquisition**
Pre-Incubation					
	None	1	95°C	10 minutes	None
Amplification					
Denaturation	Quantification	45	95°C	10 seconds	None
Annealing			50°C	35 seconds	None
Extension			72°C	1 second	Single
Cooling					
	None	1	40°C	30 seconds	None

Sensitivity of the real-time assay.

Initially serial dilutions were made of the WNV, WSLV and SINV purified cDNA. The dilutions were used as the template in qPCR reactions to determine the efficiency of the qPCR reactions. The serial dilutions ranged from $1x10^1$ copies of cDNA/µl to $1x10^7$ copies of DNA/µl. The data that was obtained was used to construct standard curves.

The information from these assays was used to determine the efficacy and error rate of the qPCR.

3.2.3. Extraction of RNA from mosquito pools

Absence of inhibitors of RT-PCR was determine by spiking a field caught mosquito known to be negative for flavivirus RNA with 1 µl of WNV RNA. RNA was subsequently extracted from the spiked mosquito homogenate using Qiazol® lysis reagent (Ambion Inc., Texas, USA) and the Qiazol® quick-start protocol. Briefly, 500 µl of Qiazol® lysis reagent was added to mosquito tissue. The tissue was homogenised in the lysis reagent using a disposable micro-pestle. To ensure that the best results are obtained the tissue needs to be homogenised thoroughly in this step. The tube was incubated at room temperature for five minutes. A total volume of 100µl of chloroform was added to the homogenate and shaken vigorously for fifteen seconds. The homogenate was incubated at room temperature for three minutes. The homogenate was centrifuged at 12 000xg for fifteen minutes at 4°C. The upper aqueous phase was carefully transferred to a new micro-centrifuge tube and 250 µl of isopropanol was added. The tube was incubated at room temperature for ten minutes. The tube was centrifuged at 12 000xg for fifteen minutes at 4°. The supernatant was aspirated and discarded. A total volume of 500 µl of 75% ethanol was added and centrifuged at 7500xg for five minutes at 4°C. The supernatant was aspirated completely and discarded. The RNA pellet was briefly air-dried. The RNA pellet was resuspended in 30 µl of RNase-free water. The RNA was stored at -80°C for use in downstream applications.

The RNA was used as template in a two-step RT-qPCR reaction. The PCR amplicon was separated with 1% gel electrophoresis and visualised under a UV transilluminator. The agarose gel separation can be seen in Figure 3.3.9. The PCR amplicon from the WNV spiked mosquito RNA extraction PCR amplicon can be seen in lane two and it has an approximate size of 414 bp.

Following the validation of the RT-PCR using mosquito extract as template, the bodies of mosquitoes that were collected in the study were pooled and stored at -20°C in RNAlater® (Sigma-Aldrich, Missouri, USA). Mosquitoes were pooled according to

species, collection site and collection date. The mosquito pools range in size depending on the number of mosquitoes collected. The pools ranged in size from one mosquito per pool to eleven mosquitoes per pool. The information for the mosquito pools can be seen in Table 3.3.2.

Mosquito head, thorax and abdomens were placed in a micro-centrifuge tube with other specimens of the same mosquito pool. The micro-centrifuge tube was briefly placed in liquid nitrogen. The mosquito tissue was broken down using a disposable micro-pestle. RNA was extracted from the mosquito tissue using Qiazol® lysis reagent (Ambion Inc., Texas, USA) and the Qiazol® quick-start protocol as mentioned above.

3.2.4. Two step RT-qPCR of mosquito pools.

The RNA extracted from the mosquito pools was reverse transcribed using Superscript™III reverse transcriptase and the FlaviF1 and Sindbis NSP2 forward primers. The resulting cDNA served as the template for the two step RT-qPCR reactions. The two step RT-qPCR's were performed using the LightCycler® TaqMan® Master kit (Roche, Manheim, Germany) according to parameters recommended by the supplier. The qPCR reactions were performed using SINV and flavivirus specific primer and probe sets in separate reactions.

3.2.5. Sequencing of amplicons and analyses of nucleotide sequences

The qPCR amplicons of samples that appeared positive in the qPCR reactions were used as the template in a PCR reaction using GoTaq®G2 hot start polymerase and the cycling conditions mentioned in Section 3.2.1.1. The PCR amplicon was separated on a 1% agarose gel through gel electrophoresis as mentioned in Section 2.2.1.2.

After visualisation the band of interest for positive samples were excised. The excised DNA bands were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, Wisconsin, USA), according to manufacturer's instructions. The purified product was sequenced bi-directionally using the BigDye® Terminator V3.1

sequencing ready reaction kit (Applied Biosystems, Foster City, CA, USA) following manufacturer's instructions as previously explained (Section 2.2.3.4) and the nucleotide sequences were edited using Chromas Pro version 1.6, aligned using Clustal Omega version 1.2.1 and sequence data retrieved from GenBank and a BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was performed.

3.3. Results

To ensure that the primers that were used in this study performed efficiently, the primer pairs were validated using transcribed RNA prepared from a region of the NS5 gene of WNV and WSLV and SINV RNA that was extracted from infected cell culture supernatant. The viral RNA served as template for RT-PCR reactions. A RT-PCR reaction was performed for SINV using the Sindbis NSP2 primer pair and SINV viral RNA. For the flaviviruses the FlaviF1/FlaviR1 primer pair was used with WNV and WSLV viral RNA.

A total of 10 µl of SINV RT-PCR amplicon was visualized after separation on a 1% agarose gel. The NSP2 primer set was used for the sample visible in lane three and it is approximately 200 bp in size according to the O'GeneRuler DNA ladder mix consisting of DNA fragments between 100 – 10 000 bp molecular weight marker. This is consistent with the expected size of 182 bp of the amplified NSP2 region (Figure 3.3.1). The negative control was run in lane 4, with no visible band. This indicates that the Sindbis NSP2 primer pair effectively amplified the SINV DNA and that the primer pair could be used successfully within the laboratory settings used in this study.

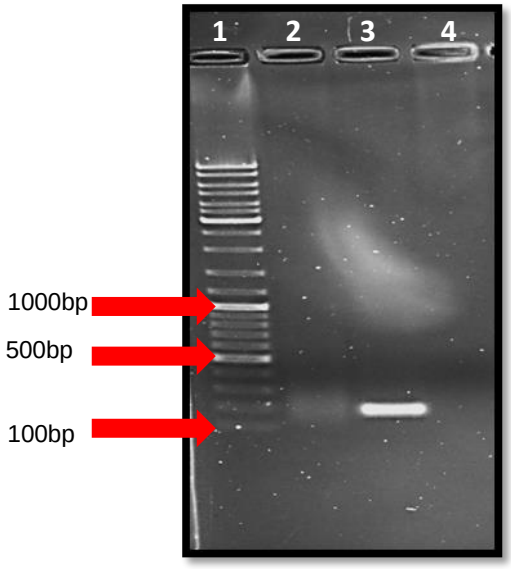

Figure 3.3.1: Agarose gel visualization of the SINV amplicon from a two-step RT-PCR reaction. Lane 1 – O'GeneRuler DNA ladder mix consisting of DNA fragments between 100 – 10 000bp (ThermoFischer Scientific, Massachusetts, USA) molecular weight marker; Lane 2 – In house primer that was compared to the NSP2 primers, but that was excluded from the study; Lane 3 – PCR amplicon amplified using NSP2 primer set and Sindbis viral RNA as template; Lane 4 – Negative control.

Similarly, for the FlaviF1/FlaviR1 primer pair amplified WNV and WSLV. RT-PCR reactions were performed on WNV and WSLV RNA and the results shown in Figures 3.3.2 and 3.3.3.

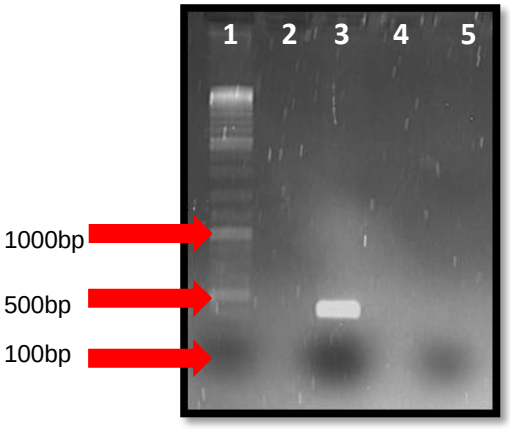

Figure 3.3.2: Agarose gel visualization of WNV amplicon from a two-step RT-PCR reaction. Lane 1 – O'GeneRuler DNA ladder mix consisting of DNA fragments between 100 – 10 000 bp (ThermoFischer Scientific, Massachusetts, USA) molecular weight marker; Lane 2 – Empty; Lane 3- PCR amplicon amplified using FlaviF1 and FlaviR1 primer set and WNV viral RNA as template; Lane 4 – Empty; Lane 5 - Negative control.

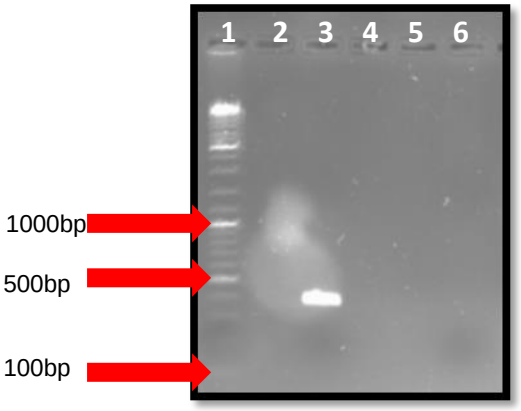

Figure 3.3.3: Agarose gel visualization of the WSLV amplicon from a two-step RT-PCR reaction. Lane 1 – O'GeneRuler DNA ladder mix consisting of DNA fragments between 100 – 10 000 bp (ThermoFischer Scientific, Massachusetts, USA) molecular weight marker; Lane 2 – Empty; Lane 3- PCR amplicon amplified using FlaviF1 and FlaviR1 primer set and WSLV viral RNA as template; Lane 4 – Empty; Lane 5 - Negative control.

The bands of interest for SINV, WNV and WSLV were excised and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, Wisconsin, USA) according to manufacturer's instructions. After purification 10 µl each of all three amplicons were visualised on a 1% agarose gel through gel electrophoresis. The SINV amplicon was run in lane two and had a size of approximately 200bp when compared to the O'GeneRuler DNA ladder mix consisting of DNA fragments between 100 – 10 000 bp molecular weight marker (Lane 1) (Figure 3.3.4). This is consistent with the expected size of 182 bp for the NSP2 region amplified by the Sindbis NSP2 primer pair (Lane 2). The WSLV amplicon was run in lane four and the WNV amplicon was run in lane six. Both these amplicons had a size of approximately 400 bp when compared to the molecular weight marker (Figure 3.3.4). This is consistent with the expected size of 414 bp of the partial NS5 gene region amplified by the FlaviF1/FlaviR1 primer pair. The purified products were run on the gel to ensure that the DNA was not lost during the purification step.

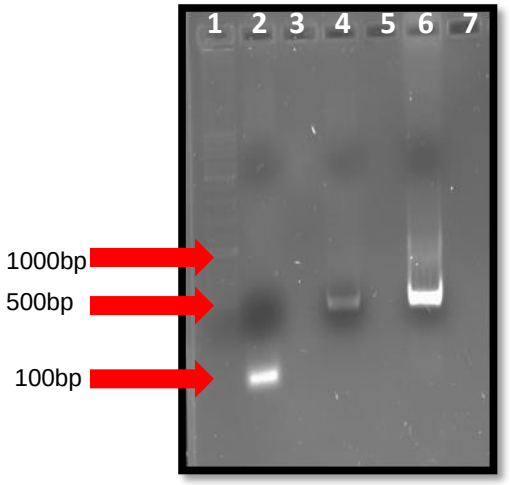

Figure 3.3.4: Agarose gel visualization of the SINV, WSLV and WNV amplicons after purification using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, Wisconsin, USA). Lane 1 – O'GeneRuler DNA ladder mix consisting of DNA fragments between 100 – 10 000 bp (ThermoFischer Scientific, Massachusetts, USA) molecular weight marker; Lane 2 – Purified SINV amplicon ; Lane 3- Empty; Lane 4 – Purified WSLV amplicon; Lane 5 – Empty; Lane 6 - Purified WNV amplicon; Lane 7 - Negative control.

The DNA concentration was determined for the purified SINV amplicon using the Quant-iT™ dsDNA BR Assay kit with the Qubit fluorometer (Life Technologies, Carlsbad, USA) according to manufacturer's instructions. The concentration was determined as 3,04 x 10^{10} copies/µl. The SINV cDNA was used directly as template for qPCR reactions.

The WSLV and WNV purified amplicons were cloned to prepare transcribed RNA for use as controls. The amount of insert that was required for the cloning was calculated as 20.7 ng of insert per 1 µl of vector. The concentrations of each WNV and WSLV amplicons was determined using the Nanodrop 2000 instrument. The concentrations were determined to be 54,2 ng/µl of insert for WNV and 47,3 ng/µl of insert for WSLV.

Three colonies were selected from each ligation reaction performed. The colonies were cultured overnight in LB broth. Plasmid preparations were purified by using PureYield™Plasmid Miniprep system. The transformants were determined to be positive through restriction enzyme digestion and confirmation of orientation of the inserted gene by performing PCR using plasmid DNA from selected colonies as template. After confirming the orientation and restriction enzyme digestion the DNA concentration was determined using Quant-iT™ dsDNA BR Assay kit with the Qubit

fluorometer (Life Technologies, Carlsbad, USA) according to manufacturer's instructions. The DNA concentration was determined for one colony of WSLV plasmid and one colony of WNV plasmid.

Purified DNA plasmid for each culture was digested using the *Not 1* restriction enzyme. A total volume of 10 µl of plasmid DNA was visualised on a 1% agarose gel following gel electrophoresis. The pGEM®-T easy vector has a size of approximately 3016 bp. The WSLV and WNV DNA have an expected size of ~414 bp. The migration patterns of the purified DNA plasmids are shown in Figure 3.3.5.

Colony one and two for WSLV did not have any bands present at 414 bp, but they had bands at ~3 000 bp (Lane 2 & 3). Colony three for WSLV and all three colonies for WNV had bands present at approximately 400 bp and 3000 bp as is to be expected (Lanes 4-7). The band for colony WSLV3 appears to be slightly larger than the other bands. It is clear that there is also a smear in this lane. This points to there being a large volume of DNA being present in lane 4, possibly causing the slower migration of this band. The negative control only had a band present at approximately 3 000 bp.

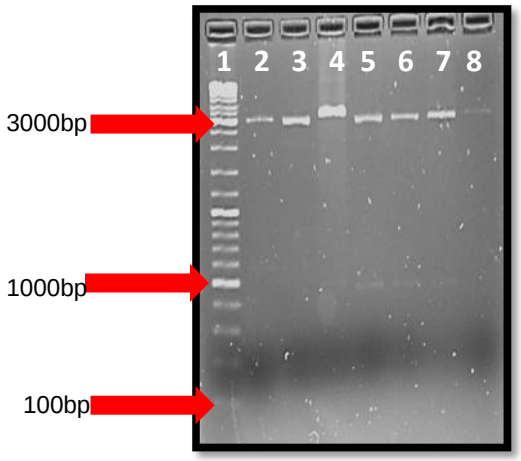

Figure 3.3.5: Agarose electrophoresis gel of restriction enzyme digestion analysis of plasmids obtained from the ligation of the flavivirus NS5 gene into pGEM®-T Easy vector. Lane 1 – O'GeneRuler DNA ladder mix molecular weight marker; Lane 2 – pGEM-WSLVNS5 colony 1 digested with *Not 1*; Lane 3 - pGEM-WSLVNS5 colony 2 digested with *Not 1;* Lane 4 - pGEM-WSLVNS5 colony 3 digested with *Not 1;* Lane 5 - pGEM-WNVNS5 colony 1 digested with *Not 1;* Lane 6 - pGEM-WNVNS5 colony 2 digested with *Not 1;* Lane 7 - pGEM-WNVNS5 colony 1 digested with *Not 1;* Lane 8 – pGEM negative control.

One colony was selected to represent each virus, based on positive restriction digestion analysis. Colony three for WSLV was the only colony for WSLV that was positive after restriction enzyme digestion. Colony one was selected for WNV, because it has the strongest DNA band following gel electrophoresis. A PCR was performed on the two selected colonies to confirm the orientation of the inserts. The PCR was performed using the SP6 reverse primer downstream of the inserted partial gene and a NS5 flavivirus specific forward primer (FlaviF1). A volume of 6 µl of PCR amplicon was separated by gel electrophoresis and visualised on a 1% agarose gel (Figure 3.3.6). This was done in duplicate for both colonies.

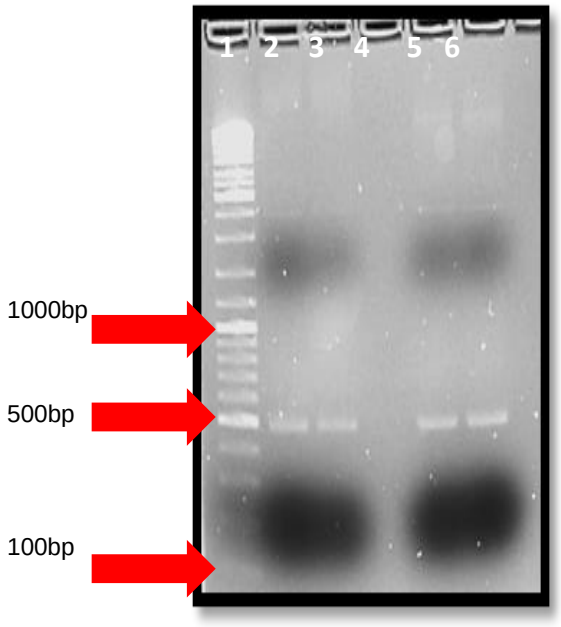

Figure 3.3.6: Agarose gel visualization of the insert DNA orientation of two selected colonies**.** Lane 1 – O'GeneRuler™ DNA ladder mix molecular marker; Lane 2 – WSLVNS5 colony 3 with an expected band size at ~414bp; Lane 3 – WSLVNS5 colony 3 repeat with an expected band size of ~414bp; Lane 4 – Empty; Lane 5 – WNVNS5 colony 1 with an expected size of ~ 414bp; Lane 6 – WNVNS5 colony 1 repeat with an expected band size of ~414bp. Bands in each lane at approximately 3 000 bp likely represent plasmid used as template.

The clones that had DNA inserted in the correct orientation were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, Wisconsin, USA). RNA was transcribed from each DNA amplicon using the SP6 MEGAscript® Kit. Subsequently an RT-PCR was performed using transcribed RNA to confirm the

application of transcribed RNA as a positive control. As shown in Figure 3.3.7, the RT-PCR reaction was able to amplify transcribed RNA that was diluted 1:100 and 1:1 000.

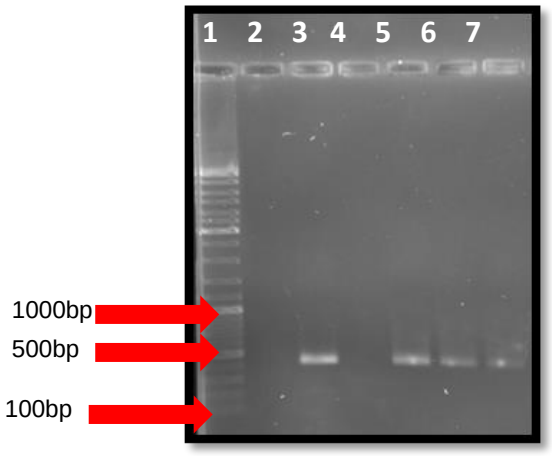

Figure 3.3.7: Agarose gel visualization of 1:100 and 1:1000 dilutions of WNVNS5 and WSLVNS5 clones. Lane 1 – O'GeneRuler™ DNA ladder mix molecular marker; Lane 2 – Negative control; Lane 3 – WNVS5 colony 1, 1:100 dilution; Lane 4 – Empty; Lane 5 – WNVNS5 colony, 1:1 000 dilution; Lane 6 – WSLVNS5 colony 3, 1:100 dilution; Lane 7 - . WSLVNS5 colony 3, 1:1 000 dilution.

Transcribed RNA was purified using the SV RNA Isolation system. A RT-PCR reaction was performed using transcribed RNA to determine that DNA was removed during the DNase step. The RT-PCR amplicons were separated through 1% agarose gel electrophoresis and visualised. As shown in Figure 3.3.8, none of the diluted samples had any DNA carryover, suggesting there was no DNA contamination of the RNA controls.

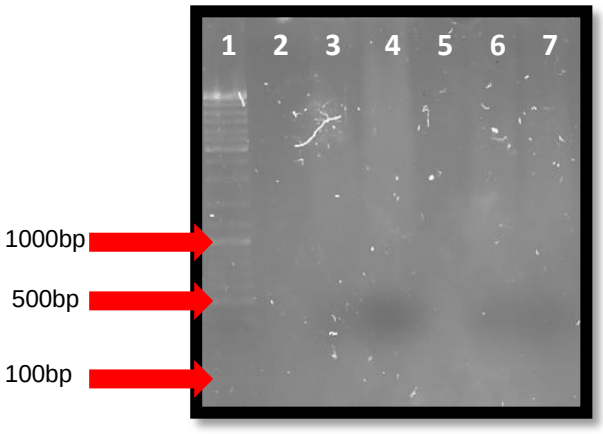

Figure 3.3.8: Gel electrophoresis visualization of PCR products from amplification of RNA controls to confirm absence of DNA contamination. Lane 1 – O'GeneRuler™ DNA ladder mix molecular marker; Lane 2 – Negative control; Lane 3 – WNVS5 colony 1, 1:100 dilution; Lane 4 – WNVNS5 colony, 1:1 000 dilution; Lane 5 – Empty; Lane 6 – WSLVNS5 colony 3, 1:100 dilution; Lane 7 - WSLVNS5 colony 3, 1:1 000 dilution.

Both the WNV and WSLV clones were positively identified through sequencing as being identical to the template RNA that was used to construct the clones. The sequence alignments for the WNV and WSLV controls can be seen in Appendix 5.4.

Transcribed RNA obtained from the WNV diluted 1:100 was selected as the positive control for the flaviviruses RT-qPCR. SINV RNA was used as the positive control for the SINV RT-qPCR. First strand cDNA was synthesised from the RNA controls and a qPCR reaction was performed on both controls. The concentration of cDNA was determined for both controls using the Quant-iT dsDNA BR Assay kit. The cDNA concentrations of the positive controls are shown in Table 3.3.1. and were used to construct the standard curves

Table 3.3.1: DNA concentrations of PCR amplicons that were constructed using RNA controls**.**

RNA control	**Concentration (copies of DNA/µl)**
WNV	1,77 x 10^{10} copies
WSLV	2,41 x 10^{12} copies
SINV	3,04 x 10^{10} copies

The RNA extraction method was tested by spiking a field caught mosquito known to be negative for flavivirus RNA with 1 µl of WNV RNA. RNA was extracted from the spiked mosquito using Qiazol® lysis reagent (Ambion Inc., Texas, USA) and the Qiazol® quick-start protocol and used as template in a two-step RT-qPCR reaction. The PCR amplicon was separated with 1% gel electrophoresis and visualised under a UV transilluminator. This was done to ensure that the RNA extraction method works suitably with the mosquito tissue. The agarose gel separation can be seen in Figure 3.3.9. The PCR amplicon from the WNV spiked mosquito RNA extraction PCR amplicon can be seen in lane two and it has an approximate size of 414 bp.

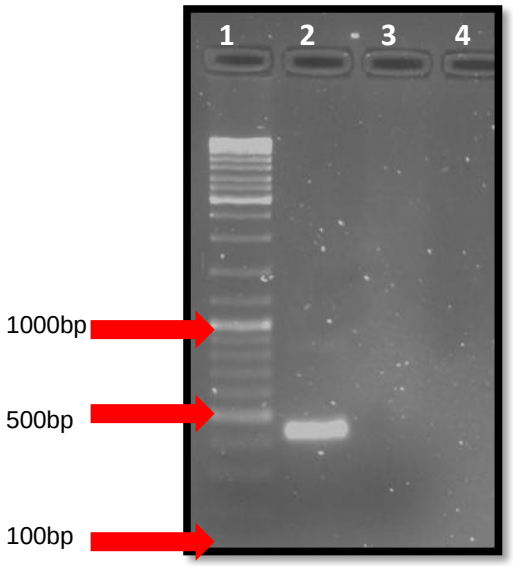

Figure 3.3.9: Gel electrophoresis results for a WNV spiked mosquito sample RNA extraction PCR amplicon. Lane 1 – O'GeneRuler™ DNA ladder mix molecular marker; Lane 2 – WNV spiked mosquito PCR amplicon.

Prior to performing RT-qPCR reactions for mosquito pools, probes were tested on RNA controls to confirm the ability of probes to detect the controls and to optimise the cycling conditions for each primer set.

The Flavi+LNA probe (Mathengtheng, 2015) and the FlaviF1 and FlaviR1 primers (Samudzi, 2008) were designed in previous studies. This probe and primer set was used to amplify the WNV RNA control DNA. The Amplification curve for the RNA control can be seen in Figure 3.3.10. The RNA control gave a positive read at the cycling conditions used with a Ct value of 21.54.

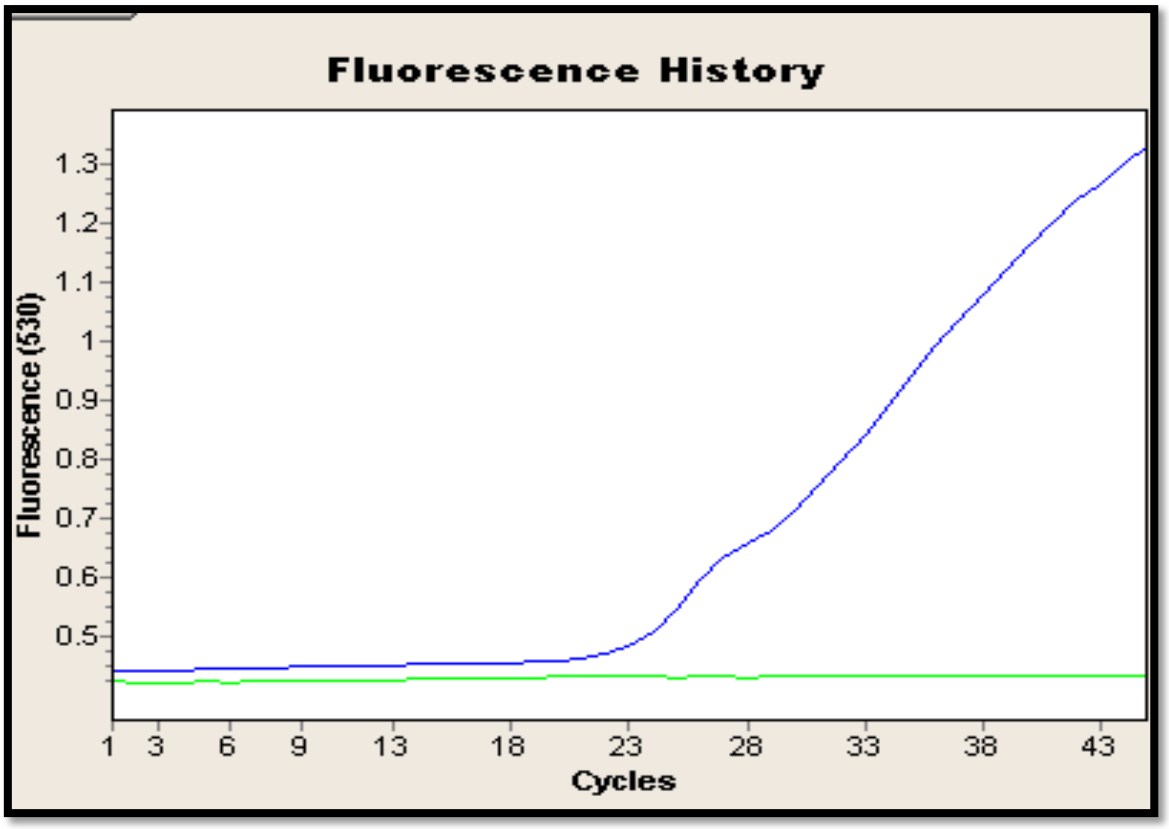

Figure 3.3.10: Amplification curves for WNV amplicon following RT-qPCR making use of a RNA control.

The NSP2 primer set and NSP2 probe were designed in a previous study (Hanekom, 2014). This primer and probe set was used to amplify the SINV RNA control DNA. The amplification curve for the control SINV RNA can be seen in Figure 3.3.11. The qPCR amplification was repeated in duplicate for the SINV RNA control samples. Both of the repeats showed a positive amplification of the RNA control. Repeat one (blue line) had a Ct value of 23,81 and the second repeat (green line) had a Ct value of 24,19.

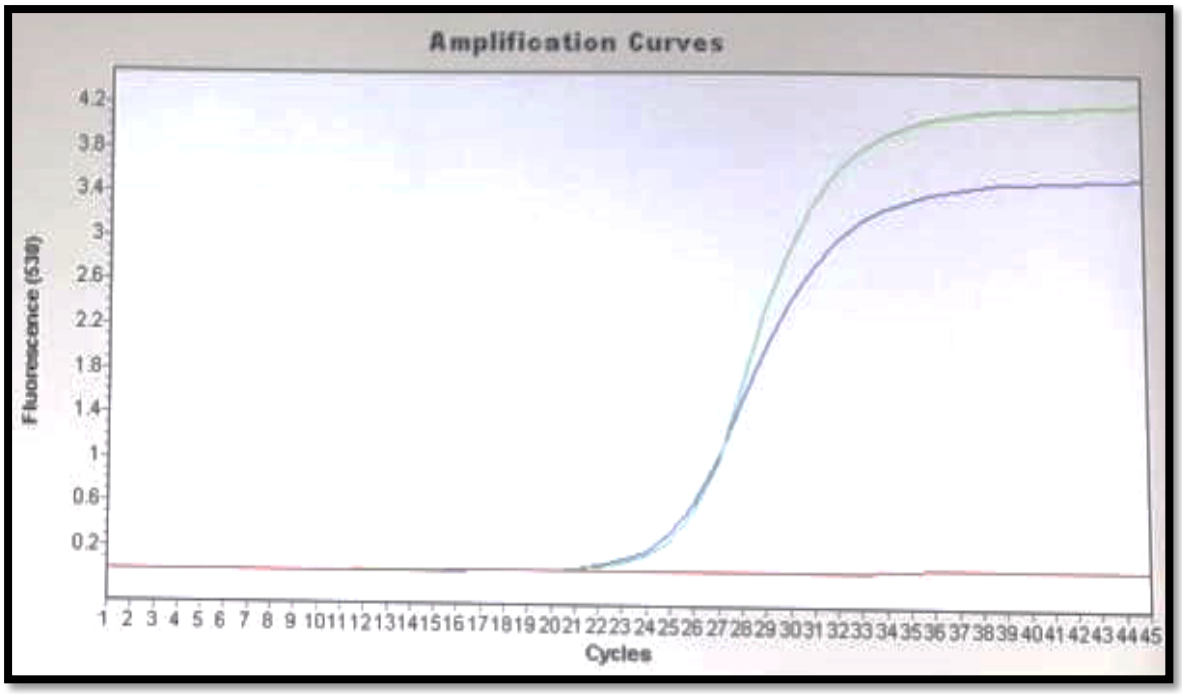

Figure 3.3.11: Amplification curves for SINV amplicon following RT-qPCR making use of an RNA control.

Following the detection of the WNV RNA control, serial 10-fold dilutions were made. DNA standards of 10^1-10^5 were used to set up the standard curve. The amplification curves for the dilution series can be seen in Figure 3.3.12. As the concentration increased the cycle threshold value decreased.

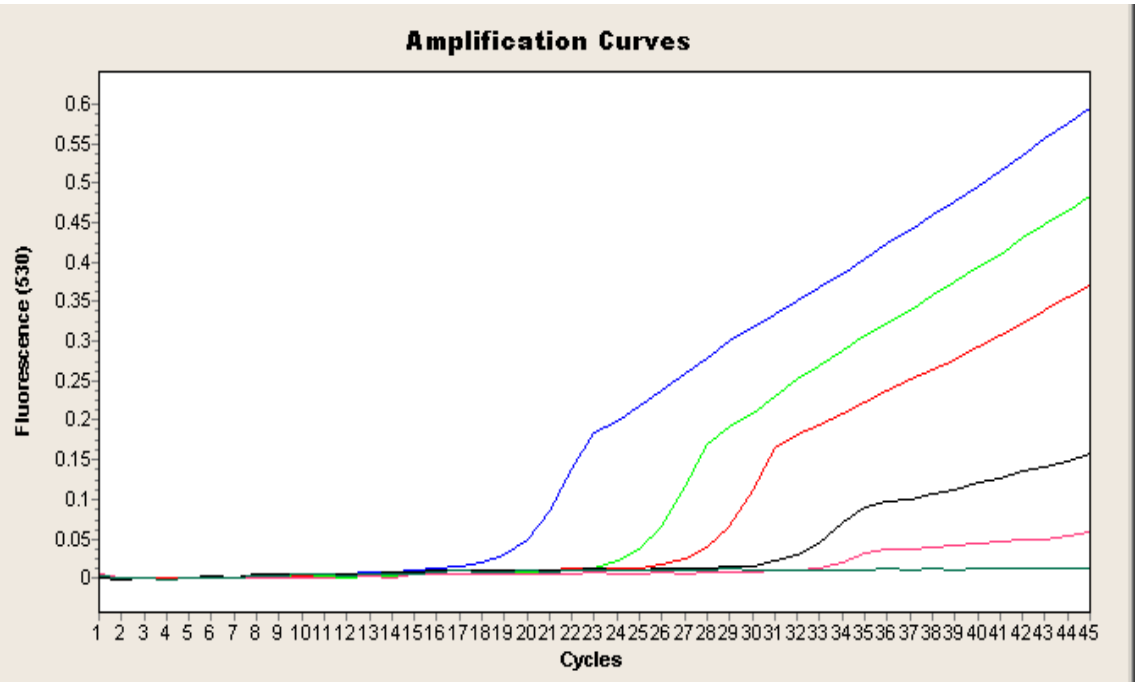

Figure 3.3.12: Amplification curves for WNV control DNA concentrations of 10^1-10^5 making use the Flavi+LNA probe.

The amplification curves were used to generate a standard curve (Figure 3.3.13). Standard curves are routinely used to indicate the efficiency of the qPCR assay. The efficiency of a qPCR reaction should be as close as possible to two, with an error rate of smaller than one. The efficiency of this PCR reaction was calculated at 2.001 with an error rate of 0.0246. The lowest level of detection for WNV was 17, 7 copies of DNA per µl.

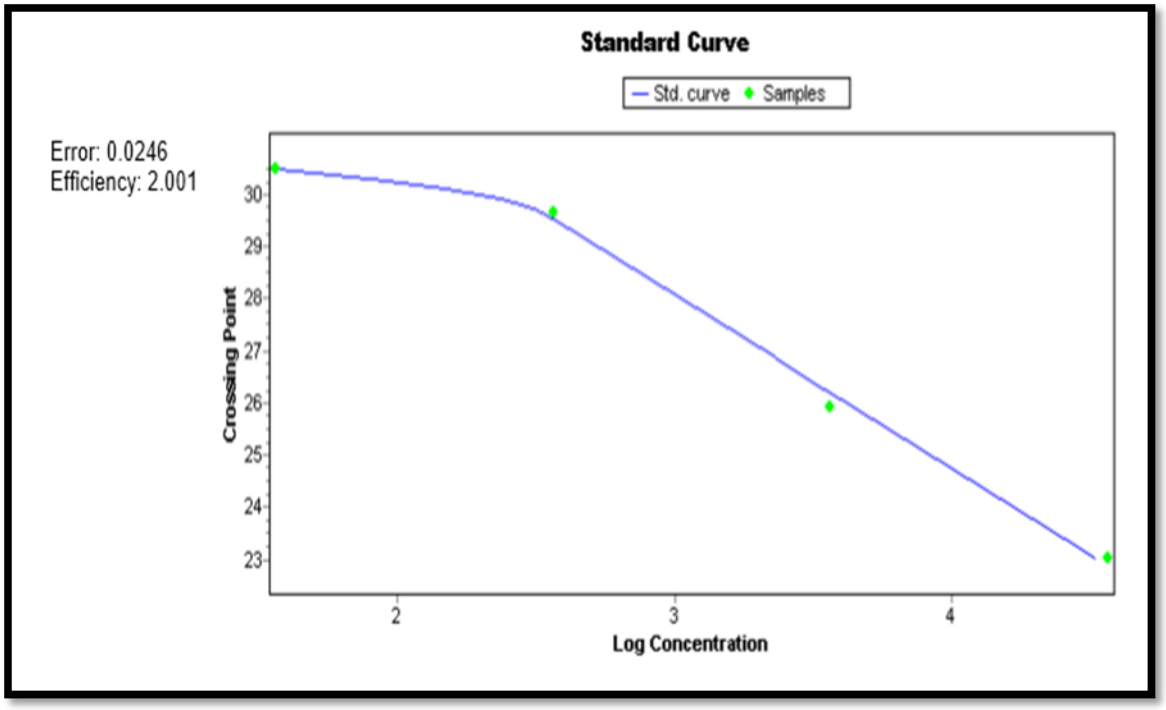

Figure 3.3.13: Standard curve generated from serial dilutions of WNV control DNA.

To confirm that the assay can successfully amplify WSLV, a RT-qPCR reaction was performed using the Flavi+LNA probe and FlaviF1/FlaviR1 primer set and the positive control RNA for WSLV (Figure 3.3.14). The positive control had a Ct value of 24.85 (blue line). This is consistent with the Ct values of the WNV control. The assay can therefore effectively be used to screen for WNV and WSLV in mosquitoes.

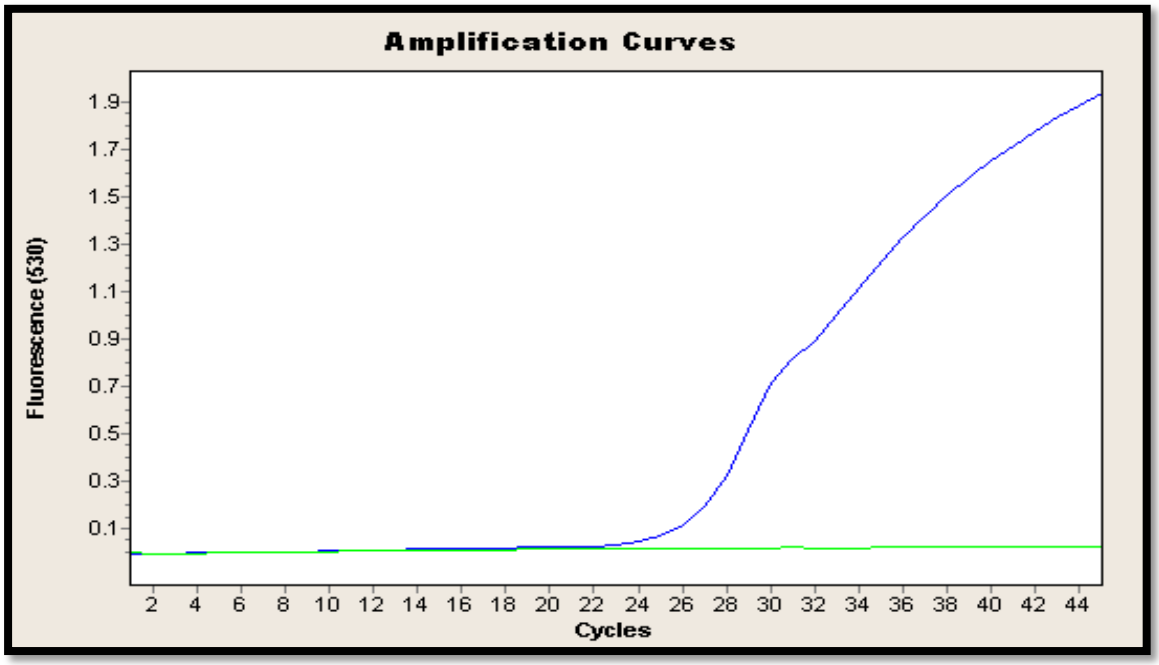

Figure 3.3.14: Amplification curves for WSLV amplicon following RT-qPCR making use of a RNA control.

Following the detection of the SINV RNA control, serial 10-fold dilutions were made. DNA standards of 10^5-10^7 were used to set up the standard curve. The amplification curves for the dilution series can be seen in Figure 3.3.15. Cycle threshold values were in agreement with the DNA standard concentrations. As the concentration increased the cycle threshold value decreased. The lowest level of detection for the SINV assay was 304 copies of DNA per µl.

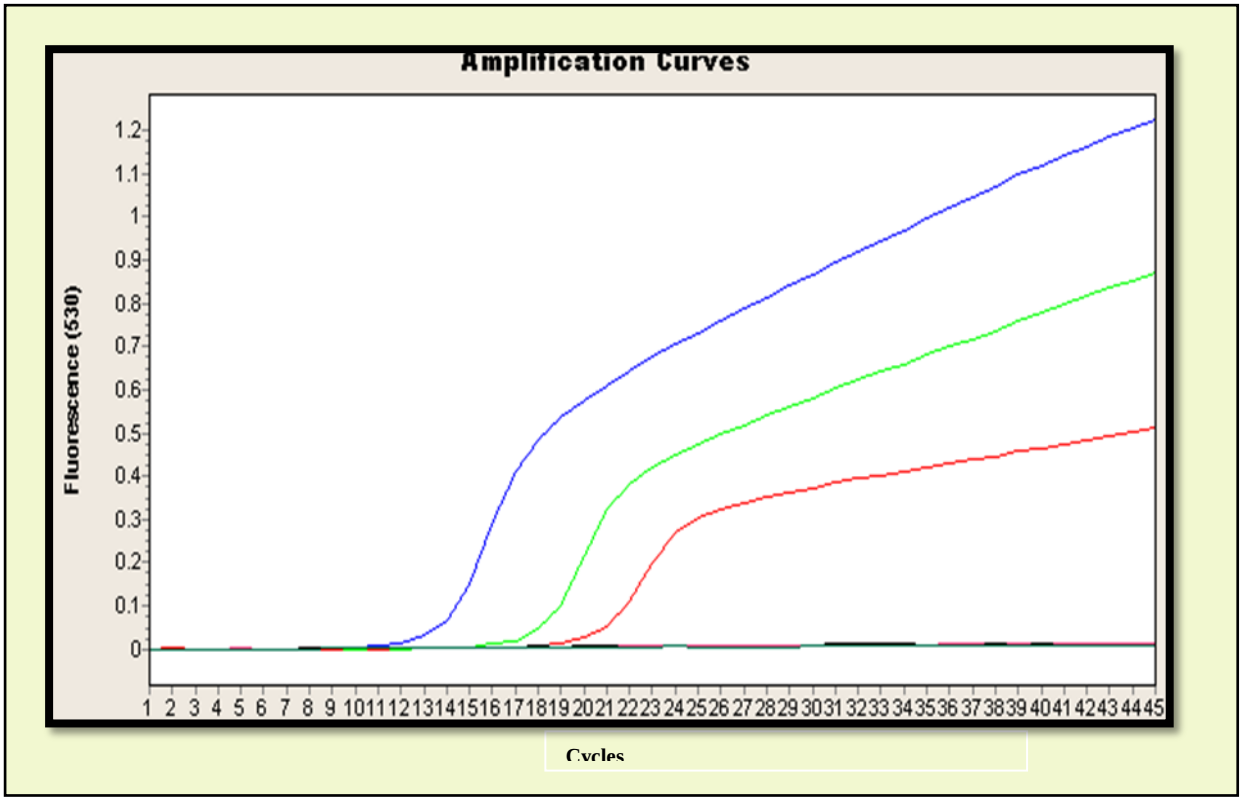

Figure 3.3.15: Amplification curves for SINV control DNA concentrations of 10^5-10^7 making use the Sindbis NSP2 probe.

The amplification curves were used to generate a standard curve (Figure 3.3.16). The efficiency of this PCR reaction was calculated at 1.978 with an error rate of 0.205.

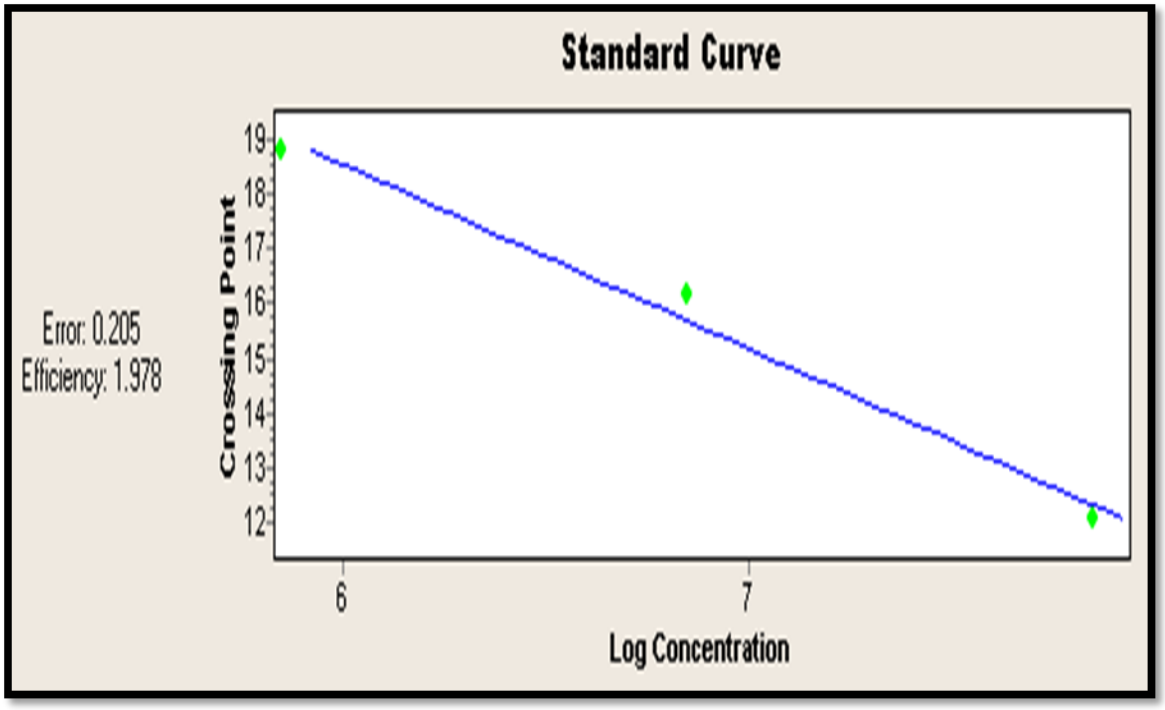

Figure 3.3.16: Standard curve generated from serial dilutions of SINV control DNA.

After it was confirmed that the RT-qPCR primers and probes amplified and detected the target DNA effectively and that the RT-qPCR reactions were efficient, RNA extracted from pooled mosquitoes were tested for the presence of flavivirus or SINV RNA. The composition of the mosquito pools is listed in Table 3.3.2. A total of 60 mosquito pools were screened.

Table 3.3.2: List of mosquito pools with species names, collection dates, collection site and the number of mosquitoes in every pool.

Pool name	Species in Pool	Collection date	Collection site	Number of mosquitoes in the pool
Pool 1	*Anopheles squamosus*	10 December 2016	Bloemfontein zoo	1
Pool 2	*Ochlerotatus harrisoni*	10 December 2016	Bloemfontein zoo	2
Pool 3	*Anopheles squamosus*	10 December 2016	Bloemfontein zoo	1
Pool 4	*Culex theileri*	10 December 2016	Bloemfontein zoo	6
Pool 5	*Culex univittatus*	11 December 2016	Free State National Botanical Gardens	1
Pool 6	*Culex theileri*	11 December 2016	Free State National Botanical Gardens	7
Pool 7	*Culex pipiens*	11 December 2016	Free State National Botanical Gardens	1
Pool 8	*Culex theileri*	28 January 2017	Bloemfontein zoo	1
Pool 9	*Ochlerotatus juppi*	29 January 2017	Free State National Botanical Gardens	1
Pool 10	*Culex theileri*	29 January 2017	Free State National Botanical Gardens	1
Pool 11	*Anopheles squamosus*	30 January 2017	Krugersdrift Dam	6
Pool 12	*Culex theileri*	27 February 2017	Bloemfontein zoo	6

Pool 13	*Culex tigripes*	27 February 2017	Bloemfontein zoo	1
Pool 14	*Culex spp.*	27 February 2017	Bloemfontein zoo	1
Pool 15	*Ochlerotatus juppi*	27 February 2017	Bloemfontein zoo	3
Pool 16	*Ochlerotatus harrisoni*	27 February 2017	Bloemfontein zoo	6
Pool 17	*Anopheles squamosus*	28 February 2017	Free State National Botanical Gardens	9
Pool 18	*Anopheles squamosus*	28 February 2017	Free State National Botanical Gardens	10
Pool 19	*Anopheles squamosus*	28 February 2017	Free State National Botanical Gardens	5
Pool 20	*Anopheles squamosus*	17 March 2017	Bloemfontein zoo	10
Pool 21	*Anopheles squamosus*	17 March 2017	Bloemfontein zoo	2
Pool 22	*Culex theileri*	17 March 2017	Bloemfontein zoo	4
Pool 23	*Aedes (Neomelaniconion) spp.*	17 March 2017	Bloemfontein zoo	1
Pool 24	*Ochlerotatus juppi*	17 March 2017	Bloemfontein zoo	2
Pool 25	*Culex univittatus*	17 March 2017	Bloemfontein zoo	3
Pool 26	*Anopheles squamosus*	18 March 2017	Free State National Botanical Gardens	9
Pool 27	*Anopheles squamosus*	18 March 2017	Free State National Botanical Gardens	10
Pool 28	*Anopheles squamosus*	18 March 2017	Free State National Botanical Gardens	10
Pool 29	*Aedes unidentatus*	18 March 2017	Free State National Botanical Gardens	2
Pool 30	*Culex theileri*	18 March 2017	Free State National Botanical Gardens	4[#]
Pool 31	*Anopheles squamosus*	18 March 2017	Free State National Botanical Gardens	10
Pool 32	*Anopheles squamosus*	18 March 2017	Free State National Botanical Gardens	10
Pool 33	*Anopheles squamosus*	18 March 2017	Free State National Botanical Gardens	10
Pool 34	*Anopheles squamosus*	18 March 2017	Free State National Botanical Gardens	10
Pool 35	*Anopheles squamosus*	18 March 2017	Free State National Botanical Gardens	10
Pool 36	*Anopheles squamosus*	18 March 2017	Free State National Botanical Gardens	10
Pool 37	*Anopheles squamosus*	18 March 2017	Free State National Botanical Gardens	10
Pool 38	*Anopheles squamosus*	18 March 2017	Free State National Botanical Gardens	10[#]
Pool 39	*Anopheles squamosus*	18 March 2017	Free State National Botanical Gardens	10
Pool 40	*Anopheles squamosus*	18 March 2017	Free State National Botanical Gardens	10
Pool 41	*Anopheles squamosus*	18 March 2017	Free State National Botanical Gardens	10
Pool 42	*Anopheles squamosus*	18 March 2017	Free State National Botanical Gardens	5
Pool 43	*Culex theileri*	19 March 2017	Krugersdrift Dam	10
Pool 44	*Culex theileri*	19 March 2017	Krugersdrift Dam	3
Pool 45	*Anopheles squamosus*	19 March 2017	Krugersdrift Dam	2
Pool 46	*Anopheles squamosus*	1 April 2017	Bloemfontein zoo	2
Pool 47	*Culex theileri*	1 April 2017	Bloemfontein zoo	4
Pool 48	*Anopheles squamosus*	2 April 2017	Free State National Botanical Gardens	9
Pool 49	*Anopheles squamosus*	2 April 2017	Free State National Botanical Gardens	10

Pool 50	*Anopheles squamosus*	2 April 2017	Free State National Botanical Gardens	10
Pool 51	*Anopheles squamosus*	2 April 2017	Free State National Botanical Gardens	1
Pool 52	*Aedes aegypti*	2 April 2017	Free State National Botanical Gardens	1
Pool 53	*Mansonia uniformis*	2 April 2017	Free State National Botanical Gardens	1
Pool 54	*Culex pipiens*	2 April 2017	Free State National Botanical Gardens	1
Pool 55	*Culex theileri*	2 April 2017	Free State National Botanical Gardens	6
Pool 56	*Anopheles spp.*	2 April 2017	Free State National Botanical Gardens	2
Pool 57	*Anopheles squamosus*	3 April 2017	Krugersdrift Dam	6
Pool 58	*Anopheles squamosus*	3 April 2017	Krugersdrift Dam	5
Pool 59	*Anopheles spp.*	17 March 2017	Bloemfontein zoo	1
Pool 60	*Anopheles spp.*	17 March 2017	Bloemfontein zoo	1
Total				316

[#] Mosquitoes were stored for pinning and therefore although they form part of the collections they did not form part of the pools.

Mosquitoes were pooled according to species, collection site and date. RNA was extracted and reverse transcribed, and the resulting cDNA served as template for qPCR reactions.

The SINV qPCR reactions were performed using a NSP2 TaqMan probe and the Sindbis NSP2 primer set targeting a region of the NSP2 gene region. The amplification curves for the SINV qPCR of the 60 mosquito pools can be seen in Figure 3.3.17.

Amplification curve A represents pool 1 – pool 10. These pools were screened using the Sindbis NSP2 primer and probe set. The only sample that amplified was the positive control (brown line) with a Ct value of 24,05. Amplification curve B represents pool 11- pool 20. These pools all tested negative for the presence of SINV. The positive control had a Ct value of 23,73. Amplification curve C represents pool 21 – pool 35. All the pools in this group tested negative for SINV using the Sindbis NSP2 primer and probe set. The positive control had a Ct value of 22,88. Amplification curve D represents pool 36 – pool 60. All the pools in this group also tested negative for SINV using the Sindbis NSP2 primer and probe set. The positive control in this group had a Ct value of 23,88. All the pools that were tested therefore gave negative results for SINV.

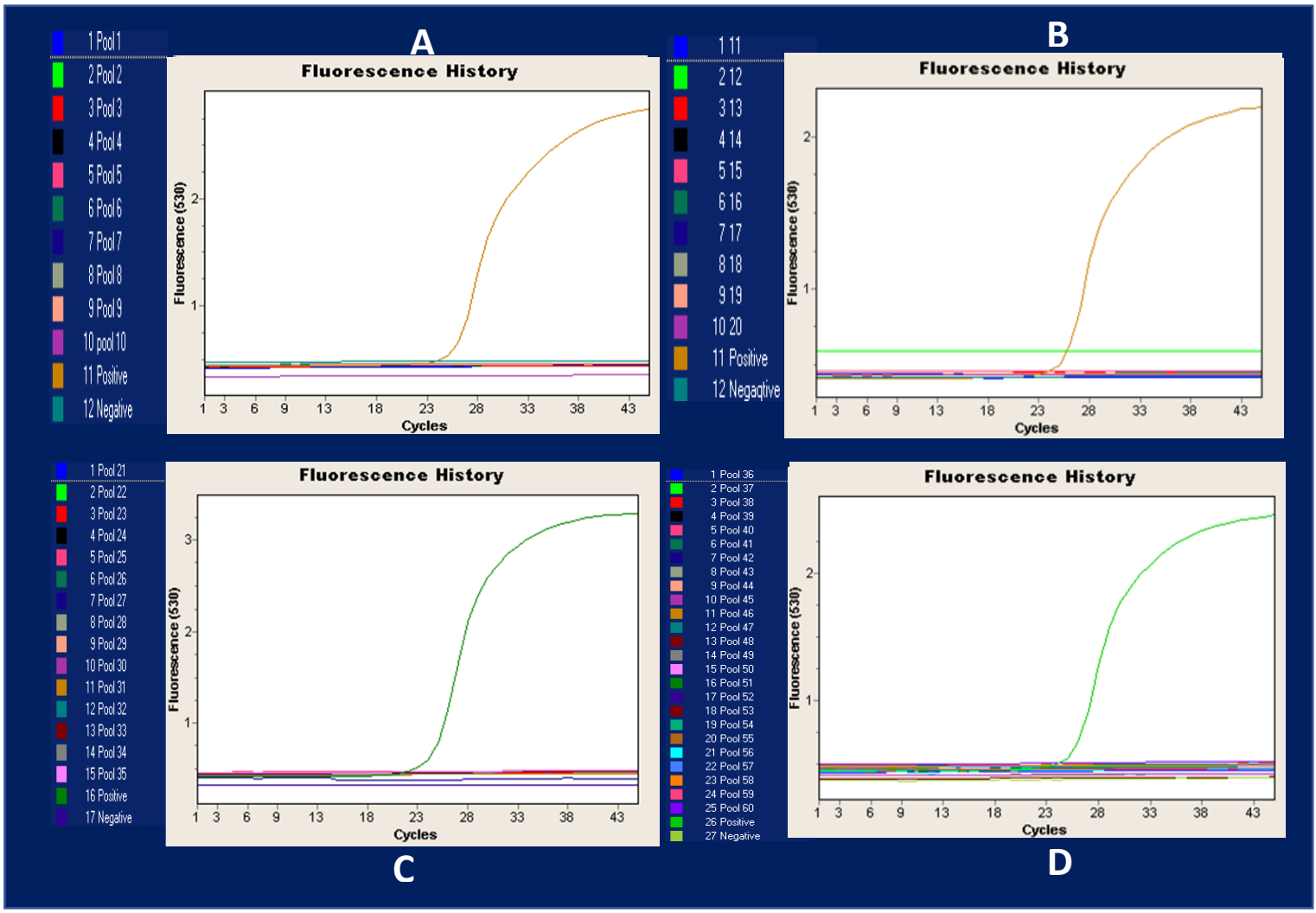

Figure 3.3.17: Amplification curves of qPCR reactions using the primer set targeting the NSP2 gene region of SINV. Figure A: Amplification curves of pool 1-10, a positive control and a negative control; Figure B: Amplification curves of pool 11-20, a positive control and a negative control; Figure C: Amplification curves of pool 21-35, a positive control and a negative control; Figure D: Amplification curves of pool 36 – 60, a positive control and a negative control.

The flavivirus qPCR reactions were performed using a Flavi+LNA probe and the FlaviF1 and FlaviR1 primer set targeting a portion of the NS5 gene. The amplification curves for the flavivirus qPCR of the 60 mosquito pools can be seen in Figure 3.3.18. Amplification curve A shows pool 1 – pool 4. One of the pools (pool one indicated in the dark blue line) showed amplification. The Ct value of the positive control was 20,05 and the Ct value of pool 1 was 33,95. Amplification curve B shows pool 5 – pool 10. All these pools were negative for WNV and WSLV. The Ct value of the positive control is 19,58. Amplification curve C shows pool 11- pool 20. Pool 15, pool 17 and pool 19

showed a small amplification curve. The Ct value for the positive control is 21,66. Pool 15 had a Ct value of 31,08, pool 17 had a Ct value of 31,01 and pool 19 had a Ct value of 31,23. Amplification curve D shows pool 21- pool 30. The pools were all negative for WNV and WSLV. The positive control had a Ct value of 20,96. Amplification curve E shows pool 31 – pool 45. The pools were all negative for WNV and WSLV. The positive control had a Ct value of 18,56. Amplification curve F shows pool 46- pool 60. The pools were all negative for WNV and WSLV. The positive control had a Ct value of 18,64.

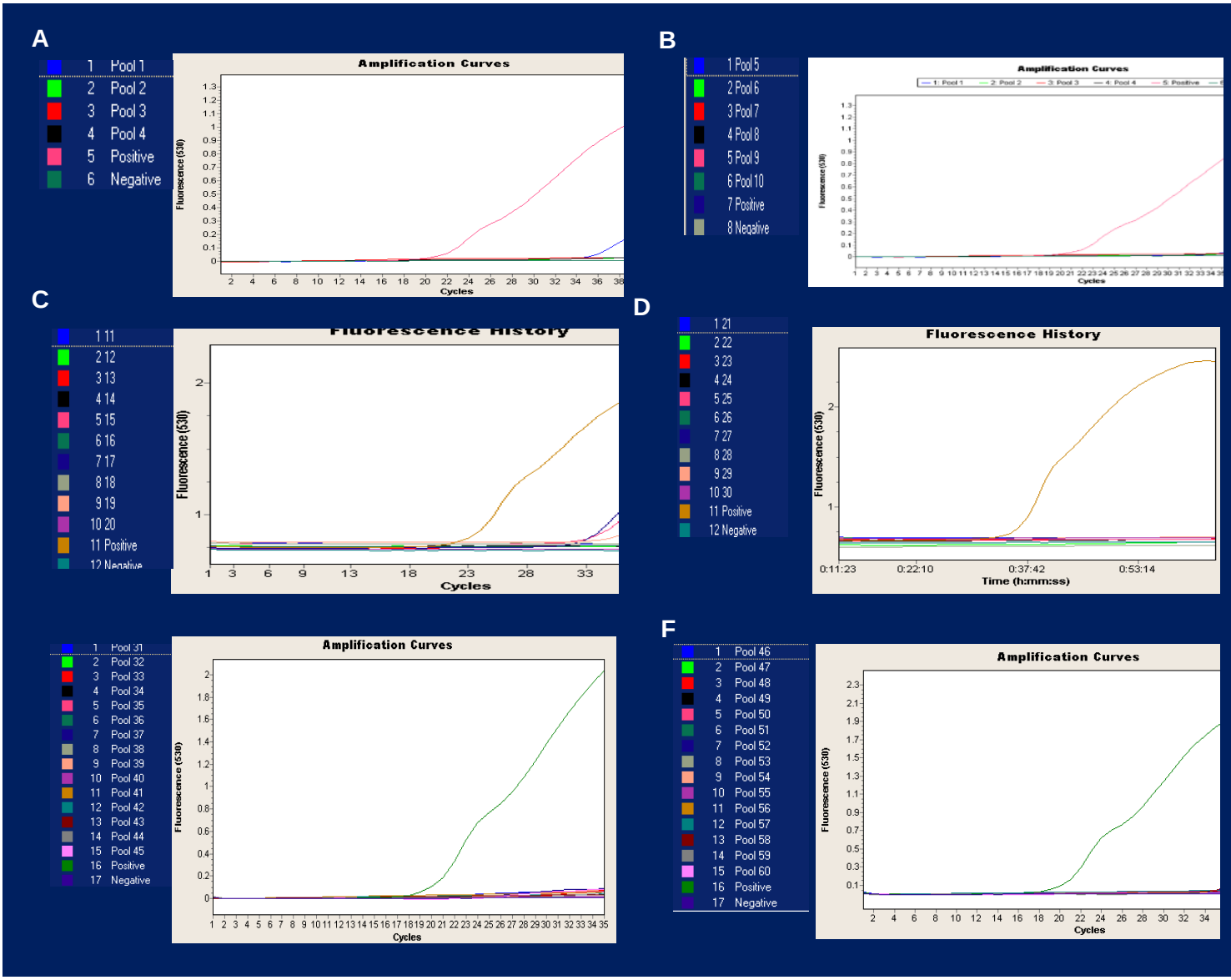

Figure 3.3.18: Amplification curves of the flavivirus qPCR reactions of 60 mosquito pools using NSP2 TaqMan probe and NSP2 forward and reverse primer set. Figure A: Amplification curves of pool 1-4, a positive control and a negative control; Figure B: Amplification curves of pool 5-10, a positive control and a negative control; Figure C: Amplification curves of pool 11-20, a positive control and a negative control; Figure D: Amplification curves of pool

21-35, a positive control and a negative control. Figure E: Amplification curves of pool 36-45, a positive control and a negative control; Figure F: Amplification curves of pool 46-60, a positive control and a negative control.

Pool 15, pool 17 and pool 19 showed a slight amplification curve. These pools were repeated and it was only pool 15 that showed amplification in the repeat. The qPCR amplicon was used as the template in a PCR reaction using GoTaq®G2 hot start polymerase and the cycling conditions mentioned in Section 3.2.1.1. The PCR amplicon was separated on a 1% agarose gel through gel electrophoresis as mentioned in Section 2.2.1.2.

As seen in Figure 3.3.19 the amplicon from pool 15 had a band at approximately 400bp, which is consistent with the expected size of 414bp of the NS5 gene region amplified by the FlaviF1/FlaviR1 primer set.

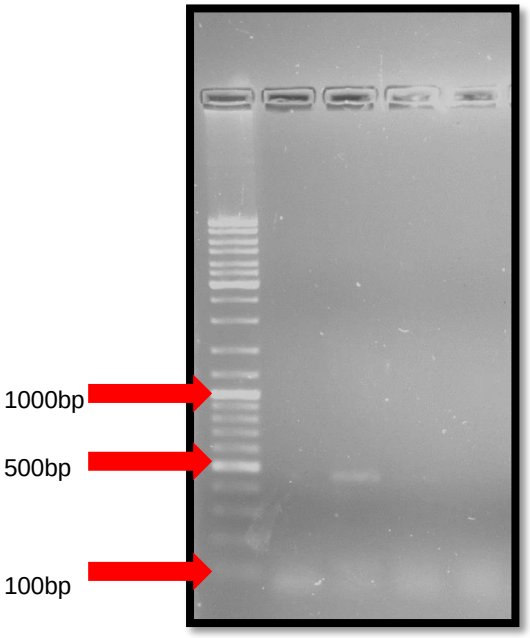

Figure 3.3.19: Agarose gel visualization of the PCR amplicons of pool 1, pool 15, pool 17 and pool 19. Lane 1 – O'GeneRuler™ DNA ladder mix molecular marker; Lane 2 – PCR amplicon of pool 1; Lane 3 – PCR amplicon of pool 15; Lane 4 – PCR amplicon of pool 17; Lane 5 – PCR amplicon of pool 19.

After visualisation the band of interest for pool 15 was excised. The excised DNA bands were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, Wisconsin, USA), according to manufacturer's instructions. The purified product was sequenced using Sanger sequencing and the nucleotide sequences were edited using Chromas Pro version 1.6, aligned using Clustal Omega version 1.2.1 and

sequence data retrieved from GenBank and a BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was performed. Sequence data analysis confirmed that the positive amplicon was contamination likely from the positive control included in the run and hence precautions were taken to prevent further contamination.

3.4. Summary

SINV RNA was used to validate the RT-PCR and RT-qPCR reactions used in this study. The efficiency of the RT-qPCR reaction was calculated as 1,978 and the error rate as 0,205 using a standard curve. RT-qPCR is the most effective and sensitive tool to quantify small amounts of nucleic acids. Standard curves are used to determine the efficiency of qPCR's, because they are reliable. The efficiency of a qPCR is defined as the fraction of target molecules that are copied in one PCR cycle (Svec *et al.,* 2015). The highest quality qPCR reactions run at an efficiency of two with an error rate of below one (Tellmann & Geulen, 2006). The qPCR was therefore proven to run efficiently.

WNV and WSLV cannot be cultured in the available BSLII laboratory,. WNV and WSLV RNA was available in a limited amount from an unrelated study and was used to prepare PCR amplicons that were cloned to provide a source. Cloned WNV and WSLV DNA as used to prepare transcribed RNA for the qPCR reactions for flaviviruses. The WNV and WSLV virus RNA was reverse transcribed into cDNA. The cDNA was used as the template in PCR reactions and the PCR amplicons were purified. The WNV and WSLV PCR amplicons were cloned into a pGEM®-T easy vector and successfully transformed. To prove that the amplicons were successfully transformed the orientation was determined through PCR, restriction digestion was performed, and the transformed DNA was sequenced. The restriction digestion reactions showed bands of the expected size on the gel electrophoresis and the sequencing reactions for both viruses were positive. The plasmid DNA was transcribed into RNA and the RNA was purified. The purified RNA was used as the positive control in downstream reactions.

The WNV RNA was used as the positive controls to test the efficiency of the flavivirus qPCR reactions used in this study. The RNA was reverse transcribed into cDNA. The

cDNA served as the template for qPCR reactions to validate the flavivirus primer and probe set. The primers were used in PCR reactions and they successfully amplified the WNV control. The efficiency of the qPCR reaction was calculated as 2,001 and the error rate as 0,0246 using standard curves. The qPCR was therefore proven to run efficiently.

Mosquitoes collected in the Bloemfontein area were sorted, pooled and screned for evidence of SINV, or flavivirus infections. Three potential positives were detected for the flaviviruses. Upon further investigation only one of the samples gave a positive result and after further testing this was proven to be contamination with the positive control.

Chapter 4 – General discussion and conclusion

Many of the mosquito species that were found in the Bloemfontein area are known vectors for WNV, WSLV, SINV and other arboviruses. This study therefore focused on development of molecular assays for screening mosquitoes for arboviral infections and barcoding mosquito species. Mosquitoes were identified through morphological and molecular means and tested for evidence of infection with selected viruses. Meteorological data for each capture site was obtained to comment on the rainfall and temperatures during the period of capture.

Mosquitoes are very important vectors of disease and understanding which species are present in an area contributes towards developing control strategies and predicting potential outbreaks of disease. The information about the abundance and diversity of mosquito species in the Bloemfontein area is very limited and an effective surveillance program in the area is lacking. Morphological identification has been seen as the gold standard of mosquito identification, but it is becoming increasingly important to use a more holistic approach when identifying mosquitoes.

Mosquitoes of the genus *Aedes* will lay their eggs singly in an area that has adequate moisture. The eggs can survive for a long time, if the environment that they are in has adequate humidity. If conditions are favourable, the eggs can even survive for a couple of years and remain dormant until they are submerged (Jupp, 1996). *Aedes* mosquitoes make use of a mechanism called instalment hatching (Jupp, 1996). Some of the eggs will hatch after the first submersion, some after the second submersion and so on. This ensures that the hatching of these mosquitoes is spread out over a larger period and this increases their chances of survival. These mosquitoes will therefore occur in smaller numbers, over a longer period of time. *Aedes* mosquitoes prefer water bodies that are deoxygenated and also contain a lot of organic material. Some of the species in this genus prefer to breed in tree holes or water retaining plant axils (Jupp, 1996; Burke *et al.,* 2010). Certain mosquitoes in this group, including *Aedes aegypti,* prefer to breed in artificial containers (Jupp, 1996; Andrew & Bar, 2013). These can include anything from tyres, water troughs, cans or artificial pools. *Aedes aegypti* is well adapted to live in urban areas (Burke *et al.* 2010). Mosquitoes

in this genus feed readily on non-human primates and humans. Most of these mosquitoes are diurnal feeders (Jupp, 1996).

Mosquitoes in the sub-genera *Neomelaniconion* and *Ochlerotatus* will lay their eggs in grassland habitats in areas that get flooded after heavy rains. These include areas like pools in river floodplains, dams, vlei areas and pans. The eggs can survive for several years in areas that have a few centimetres of moist soil and are able to remain dormant until the area is flooded again. Some of the mosquitoes in these sub-genera that can reach the highest densities include *Aedes unidentatus* and *Ochlerotatus juppi* (Jupp, 1996).

Culex univittatus mosquitoes were collected at both the Bloemfontein Zoo and the Free State National Botanical Gardens. The zoo has multiple small pans that have high levels of organic material and the Botanical Gardens has an ephemeral pan that covers an area of grass. *Culex univittatus* rarely feeds on humans and they are only endophagic to a very lesser extent. These mosquitoes act as the vectors for these viruses, but they tend not to feed on humans (Jupp, 1996) which can explain why epidemics of Sindbis and West Nile in humans are rare.

Culex theileri was the most abundant *Culex* species in all three sampling sites and the second most abundant species in the Bloemfontein area. Overall the number of *Culex* spp. mosquitoes captured were significantly lower than those of *Anopheles* mosquitoes. *Culex theileri* can utilize a wide range of breeding sites (Simsek, 2004) and will breed in any area that has a permanent source of water (Jupp, 1996). The larvae of this species can be found in flooded meadows, stagnant water and pools. They can also be found in artificial containers in the Free State, contrary to the findings of Simsek (2004), who failed to report *Culex theileri* from garden pools and drinking troughs in Turkey. The larvae of these mosquitoes can also withstand highly polluted water (Simsek, 2004; Demirci *et al.,* 2012, Demirci *et al.,* 2014). *Culex theileri*, unlike *Culex univittatus*, feed primarily on mammals and to a lesser extent on birds (Martinez-de-la-Puente *et al.,* 2012). They are known to feed on humans and are known to be endophagic, which means that they will frequently enter human habitations (Jupp, 1996; Simsek, 2004). These mosquitoes can be found in high densities (Simsek, 2004) *Culex* (*Lutzia*) *tigripes* have predatory larvae and these larvae will prey on the larvae of other mosquitoes (Jupp, 1996). Mosquitoes of the sub-genera *Culex* mostly lay their

eggs in permanent water bodies. The eggs of these mosquitoes cannot tolerate desiccation. These mosquitoes therefore lay their eggs in small rafts on the water surface. The rafts can contain anything from 100-300 eggs (Jupp, 1996). Many of the species in the sub-genus *Culex* primarily feed on birds, but they also feed on mammals (Jupp, 1996, Demirci *et al.,* 2014). *Culex pipiens* mosquitoes prefer water bodies that are rich in organic material. This organic material can be either human effluent or natural organic material (Jupp, 1996). *Culex univittatus* will breed in water bodies that are temporary or semi-permanent, such as pans or pools (Jupp & McIntosh, 1967; Jupp, 1996). These mosquitoes tend to breed in water that is nutrient poor and contains emergent vegetation (Jupp & McIntosh, 1967; Uejio *et al.,* 2012).

Anopheles squamosus was the most abundant mosquito found in the Bloemfontein area, being found in all three of the sampling sites. *Anopheles squamosus* is largely neglected in the *Anopheles* group, because they are considered as mainly zoophilic mosquitoes,that feed primarily on domesticated animals, especially bovines and goats. (Norris & Norris, 2015; Nyirakanani *et al.,* 2017). *Anopheles squamosus* tends to not feed on humans in the presence of other vertebrate hosts but increased anthropophily has been shown in Zambia. The human biting indices of *Anopheles squamosus* is very low in Burkina Faso, Ghana, Kenya, Nigeria and Zimbabwe and they are known to be primarily zoophagic in South Africa (Norris & Norris, 2015; Nyirakanani *et al.,* 2017). *Anopheles squamosus* therefore shows differing levels of anthropophily and anthropophagy in different geographic areas (Fornadel *et al*., 2011; Nyirakanani *et al.,* 2017). *Plasmodium falciparum* sporozoites have been found in dissections of *Anopheles squamosus* in Tanzania and Zimbabwe. *Anopheles squamosus* is currently seen as a secondary vector for malaria (Norris & Norris, 2015; Nyirakanani *et al.,* 2017). Secondary vectors are exophilic or exophagic and they can therefore potentially sustain malaria transmission after the endophilic and endophagic vectors have been reduced by indoor mosquito control measures. *Anopheles squamosus* will show foraging behaviour in the early evening with moderate biting throughout the night. Increasing population densities have been seen for these mosquitoes. This might lead to an increased biting rate of these mosquitoes on human hosts. This in turn will further solidify these mosquito's role as a secondary mosquito vector of malaria (Fornadel *et al*., 2011). RVFV has also been detected in *Anopheles squamosus* mosquitoes (Nepomichene *et al.,* 2018). Due to the fact that these

mosquitoes are more frequently biting humans, increased densities and increased evidence of these mosquitoes as potential disease vectors it is very important that these mosquitoes are studied in depth. These mosquitoes have not been studied extensively in the Bloemfontein area and future research of these mosquitoes should focus on human biting rate and mosquito densities in the Bloemfontein area and in South Africa as a whole to see what the role is of this mosquito as a possible disease vector in South Africa.

There is evidence that *Anopheles squamosus* might have a cryptic sibling species (Fornadel *et al*., 2011). This has implications for mosquito identification. Morphological identification might not be able to distinguish this mosquito from a sibling species. In this case molecular sequencing might be essential in determining the presence of such species. Sixteen sequences were obtained for species that were positively identified as *Anopheles squamosus* through morphology. Future studies will focus on obtaining sequence data from more *Anopheles squamosus* mosquitoes. Positive sequences will be submitted to databases like GenBank or BOLD.

One specimen of the genus *Mansonia* was identified using morphological keys: *Mansonia uniformis*. These mosquitoes tend to be found in pans or lakes where there are significant amounts of floating vegetation and are unusual in that they are one of the few species that can tolerate flowing water for oviposition and larval and pupal development. The adult female mosquito attaches her eggs in clusters on the underside of leaves and on the roots and stems of floating vegetation. The eggs bear a sharp point that can be inserted into the plant parenchyma. The larva has a sharply pointed respiratory siphon and the pupa has similarly pointed respiratory trumpets that allow anchoring onto water plants. Immature stages of *Mansonia* spp. can remain submerged because they obtain oxygen directly from plant roots. This is consistent with the Free State National Botanical Gardens environment that this specimen was captured in. This area has a semi-permanent dam that has a variety of aquatic plants. These mosquitoes will aggressively feed on humans and they are endophagic. They are mainly nocturnal, but diurnal activity has also been reported. They have quite a broad host range including humans, cattle, birds, monkeys, rock hyraxes, rodents and reptiles (Jupp 1996).

Four specimens of *Anopheles*, one specimen of *Aedes (Neomelaniconion)* and one specimen of the genus *Culex* could not be identified to species level using morphological keys or molecular barcoding. *Aedes aegypti, Culex theileri, Anopheles squamosus* and *Mansonia uniformis* were positively confirmed using genetic barcoding. *Ochlerotatus juppi* and *Ochlerotatus harrisoni* mosquitoes were identified morphologically and although sequence data was obtained in the study there were no sequences available on the public databases for comparison. Additional mosquitoes will be analysed in future studies to confirm the sequence determined in this study and the data submitted to GenBank or BOLD. In future studies it would be worthwhile to provide more barcoding primers to get genetic sequences for the South African mosquito species that do not have sequence data available. Variations in species identification is normally seen either because of a low level of morphological identification skills or due to a large variation in molecular sequencing of individual mosquitoes. Variation in molecular sequencing was seen in *Anopheles gambiae*. In the ITS2 region there is a considerable molecular variation. This is most likely because the protocols for sequencing have not been optimized for other anopheline species. This may lead to confusing results. Some specimens also show some levels of non-specific amplification. It is very important to note that molecular and morphological techniques should always be used in conjunction. Standard molecular techniques should always be performed and then results should be confirmed and strengthened through molecular techniques (Erlank, *et al*., 2018). It is clear that newer molecular techniques should be optimised for all mosquito species to ensure reliable results. Future research needs to be done to determine the level of variation in sequencing results for different mosquito species in different geographic regions.

It is clear to see from the meteorological data of the Bloemfontein area that the average summer and winter temperatures are gradually increasing with above average temperatures being experienced since 2015 (Appendix 5.2). Above average summer temperatures have an effect on mosquito life cycles and have also been connected to the outbreaks of arboviruses in South Africa. Over the study period Bloemfontein experienced drought conditions with below average rainfall. Outbreaks of mosquito-borne diseases have been associated with high rainfall. To determine the effect of temperature and rainfall on the mosquito diversity and abundance in the Bloemfontein area continued collections of mosquitoes need to be performed.

WNV, WSLV and SINV are known to circulate in the inland plateau of South Africa and it is clear that competent vectors for these viruses do occur in this area. Although the 60 pools of mosquito tested negative for all three arboviruses, the small sample sizes of the mosquitoes tested were a serious limitation.

WNV continually circulated in many locations worldwide and naturally occurs in South Africa (Uejio, *et al.,* 2012). WNV neutralising antibodies have been detected in many areas of South Africa. The virus has been isolated on the inland plateau and the Kwa-Zulu Natal coast. Neutralising antibodies were detected in humans on the inland plateau, with 17.1% of humans testing positive in the Karoo region and 8% in the Highveld region (Jupp, 2001). Annually, sporadic cases of West Nile are reported in the Highveld area in the summer months. In 1974 there was an outbreak of WNV in the Karoo region and an epizootic with more human cases in the Highveld region in 1984 (Jupp, 2001, Venter, 2018).

A surveillance program between 1956 and 1980 isolated WNV from *Culex univittatus*, *Culex theileri*, *Culex pipiens* and *Ochlerotatus caballus*. Between 1956 and 1980 a total of 283 808 mosquitoes were collected and only 128 isolations were made from *Culex univittatus* (70 037 specimens = 0.18%), six isolations from *Culex theileri* (82 995 specimens = 0.007%), one isolation from *Culex pipiens* (128 967) and one isolation from *Ochlerotatus caballus* (1 809) (Jupp, 2001; Chevaler *et al.*, 2004). In South Africa, *Culex theileri* mosquitoes are known to be naturally infected with SINV (Simsek*,* 2004; Demirci *et al.,* 2012; Demirci *et al.,* 2014). Both *Culex univittatus* and *Culex theileri* were found in the Bloemfontein area but the small percentage of infections shown above underlines the very large sample sizes that are required in surveillance activities, something the current project lacked.

To get a clearer picture of the presence of WNV in the mosquitoes of the Bloemfontein area more mosquito collections over a longer period of time is needed. *Culex univittatus* has a low infection threshold and a high transmission rate (97%), while *Culex theileri* has a low infection threshold that is comparable to that of *Culex univittatus*, but a low transmission rate. This difference can be attributed to the behaviour of these two mosquito species. *Culex univittatus* is mainly ornithophilic and tends to feed on birds roosting in long grass and papyrus reeds, while *Culex theileri* feeds mostly on mammals, but will feed on nearly any vertebrate, including birds and

humans. It is for this reason that *Culex theileri* acts as a bridging vector and infections in these mosquitoes will lag behind that of *Culex univittatus*. Although *Culex univittatus* is the main vector of WNV in South Africa, they tend not to feed on humans, and this is seen as a limiting factor in human infections. Humans also act as dead-end hosts with a low viraemia (Jupp, 2001). With *Culex theileri* being the main representative of the *Culex* genus in the Bloemfontein area, WNV isolations are expected to be low. WNV has not been isolated in any other species of mosquito on the inland plateau (Jupp, 2001).

SINV has the same ecology in South Africa as WNV and outbreaks of the two viruses seem to coincide. The outbreaks of WNV and SINV in South Africa were accompanied by above average summer temperatures and exceptionally good summer rains (Jupp, 2001). Every year cases of SINV are reported in the Highveld of South Africa (Uejio, *et al.,* 2012; Venter, 2018). *Culex* mosquitoes are implicated in SINV infections, with *Culex univittatus* as the main vector (Uejio, *et al.,* 2012).

WSLV is widespread on the African continent and in South Africa only *Aedes* and *Ochlerotatus* species have been implicated in transmission. These include *Aedes (Neomelaniconion) circumluteolus*, *Aedes mcintoshi/luridus* and *Ochlerotatus juppi/caballus* (Jupp, 2005). WSLV outbreaks are sporadic, with a few years between outbreaks. Between 1955 and 2011 a total of 35 human WSLV isolations were found, most of them in South Africa, Central African Republic and Senegal (Diagne, *et al*., 2017).

Another mosquito-borne virus that circulates in South Africa is Rift Valley fever. This virus has been isolated from numerous species, including *Aedes circumluteolus, Aedes mcintoshi*, *Ochlerotatus juppi/caballus* and *Culex theileri*. This virus was not tested for in this study, but in future studies it would be wise to include Rift Valley fever in the screening process.

In this study no traces of arboviruses were found in the mosquitoes that were caught in the Bloemfontein area. Due to the fact that the infectivity rates of mosquitoes are very low and that the number of mosquitoes caught were limited, it is to be expected that no viruses were detected. The methods that were used in this study, however, are effective to screen for arboviruses and can be successfully applied to larger

surveillance programmes in the future. Future studies should also focus on a larger sample size in a wider geographical area.

A few limitations were identified in the study. Mosquito trapping methods focussed on mainly nocturnal species, so in future studies it might be worthwhile to include other trapping methods that are more appropriate for collecting diurnal mosquitoes. The sample size of mosquitoes was small and to get more conclusive data continued mosquito collections will be needed in more locations around the Bloemfontein area.

The COI barcoding region was able to effectively confirm the identity of four species of mosquito. The other species had limited or no sequence data available, and this study will provide barcode sequences to GenBank that can be used as a baseline in other studies. Continued surveillance will also assist with drawing comparisons between weather data and mosquito abundance.

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5. Appendix

5.1 Barcoding sequences for selected mosquito species in the Bloemfontein area

Sequence data of selected mosquito species collected in the Bloemfontein area for the period of December 2016-April 2017.

The sequences for the mosquitoes identified as *Aedes aegypti* are shown below.

VBDF 14-04 is a mosquito specimen that was identified as *Aedes aegypti* using morphological identification.

TAGTCGGAACTTCTCTAAGAATTTTAATTCGTGCTGAACTTAGCCACCCTGGTAT
ATTTATTGGGAATGACCAAATTTATAATGTAATTGTAACAGCTCATGCATTTATTA
TAATTTTCTTTATAGTAATGCCAATTATAATTGGAGGATTTGGAAATTGATTAGTT
CCTTTAATATTAGGAGCCCCTGATATAGCTTTCCCTCGAATGAATAATATAAGTT
TTTGAATACTACCTCCTTCATTGACTCTTCTATTATCAAGCTCAATAGTAGAAAAT
GGAGCAGGAACTGGGTGAACAGTTTATCCTCCTCTCTCTTCAGGAACAGCTCAT
GCTGGGGCTTCTGTTGATTTAGCTATTTTTTCTCTTCATTTAGCTGGAATTTCCT
CAATTTTAGGGGCAGTAAATTTTATTACAACTGTAATTAATATACGATCGTCAGG
GATTACTTTAGATCGACTACCCTTATTTGTTTGATCTGTAGTTATTACAGCTATCT
TATTACTTCTTTCTCTTCCTGTTTTAGCTGGAGCTATTACTATATTATTAACAGAC
CGAAACTTAAATACATCTTTCTTTGATCCAATCGGAGGGGGAGACCCTA

The sequences for different mosquitoes that were identified morphologically as *Anopheles squamosus* are shown below.

VBDF 10-01 is a mosquito specimen that was identified as *Anopheles squamosus* using morphological identification.

GGAACTTTATACTTTATTTTCGGTGCTTGAGCTGGAATAGTAGGTACTTCTTTAA
GTATTCTAATTCGAGCTGAATTAGGACATCCAGGAGCATTTATTGGAGATGATC
AAATTTATAATGTAATCGTAACTGCTCACGCTTTTATTATAATTTTTTTTATAGTAA
TACCAATTATAATTGGGGGATTCGGAAATTGACTAGTTCCTTTAATATTAGGAGC
CCCAGATATAGCTTTCCCTCGAATAAATAATATAAGTTTTTGAATACTTCCTCCAT
CTTTAACATTACTTATCTCTAGTAGTATAGTAGAAAACGGAGCTGGAACAGGATG
AACAGTTTATCCTCCTCTTTCTTCTGGAATTGCTCACGCAGGAGCTTCAGTAGAT
TTAGCTATTTTCTCTCTTCATTTAGCAGGGATTTCATCAATTTTAGGAGCTGTAAA
TTTTATTACTACAGTAATTAATATACGATCTCCTGGAATTACTCTAGATCGAATAC
CTTTATTTGTTTGATCTGTAGTAATTACAGCTGTATTATTATTATTATCTTTACCTG
TATTAGCAGGAGCAATTACAATATTATTAACTGATCGAAATTTAAATACATCATTC
TTCGACCCTGCTGGAGGTGGAGACCC

VBDF 10-02 is a mosquito specimen that was identified as *Anopheles squamosus* using morphological identification.

GGTGCTTGAGCTGGAATAGTAGGTACTTCTTTAAGTATTCTAATTCGAGCTGAAT
TAGGACATCCAGGAGCATTTATTGGAGATGATCAAATTTATAATGTAATCGTAAC
TGCTCACGCTTTTATTATAATTTTTTTTATAGTAATACCAATTATAATTGGGGGAT
TCGGAAATTGATTAGTTCCTTTAATATTAGGAGCCCCAGATATAGCTTTCCCTCG
AATAAATAATATAAGTTTTTGAATACTTCCTCCATCTTTAACATTACTTATCTCTAG
TAGTATAGTAGAAAACGGGGCTGGAACAGGATGAACAGTTTATCCCCCTCTTTC
TTCTGGAATTGCTCACGCAGGAGCTTCAGTAGATTTAGCTATTTTCTCTCTTCAT
TTAGCAGGGATTTCATCAATTTTAGGAGCTGTAAATTTTATTACTACAGTAATTAA
TATACGATCTCCTGGAATTACTCTAGATCGAATACCTTTATTTGTTTGATCTGTA
GTAATTACAGCTGTATTATTATTATTATCTTTACCTGTATTAGCAGGAGCAATTAC
AATATTATTAACTGATCGAAATTTAAATACATCATTCTTCGACCCTGCTGGAGGT
GGAGACCC

VBDF 10-03 is a mosquito specimen that was identified as *Anopheles squamosus* using morphological identification.

TTGAGCTGGAATAGTAGGTACTTCTTTAAGTATTCTAATTCGAGCTGAATTAGGA
CATCCAGGAGCATTTATTGGAGATGATCAAATTTATAATGTAATCGTAACTGCTC
ACGCTTTTATTATAATTTTTTTTATAGTAATACCAATTATAATTGGGGGATTCGGA
AATTGATTAGTTCCTTTAATATTAGGAGCCCCAGATATAGCTTTCCCTCGAATAA
ATAATATAAGTTTTTGAATACTTCCTCCATCTTTAACATTACTTATCTCTAGTAGT
ATAGTAGAAAACGGGGCTGGAACAGGATGAACAGTTTATCCCCCTCTTTCTTCT
GGAATTGCTCACGCAGGAGCTTCAGTAGATTTAGCTATTTTCTCTCTTCATTTAG
CAGGGATTTCATCAATTTTAGGAGCTGTAAATTTTATTACTACAGTAATTAATATA
CGATCTCCTGGAATTACTCTAGATCGAATACCTTTATTTGTTTGATCTGTAGTAA
TTACAGCTGTATTATTATTATTATCTTTACCTGTATTAGCAGGAGCAATTACAATA
TTATTAACTGATCGAAATTTAAATACATCATTCTTCGACCCTGCTGGAGGTGGAG
ACCC

VBDF 10-04 is a mosquito specimen that was identified as *Anopheles squamosus* using morphological identification.

ATAGTAGGTACTTCTTTAAGTATTCTAATTCGAGCTGAATTAGGACATCCAGGAG
CATTTATTGGAGATGATCAAATTTATAATGTAATCGTAACTGCTCACGCTTTTATT
ATAATTTTTTTTATAGTAATACCAATTATAATTGGGGGATTCGGAAATTGATTAGT
TCCTTTAATATTAGGAGCCCCAGATATAGCTTTCCCTCGAATAAATAATATAAGT
TTTTGAATACTTCCTCCATCTTTAACATTACTTATCTCTAGTAGTATAGTAGAAAA
CGGGGCTGGAACAGGATGAACAGTTTATCCCCCTCTTTCTTCTGGAATTGCTCA
CGCAGGAGCTTCAGTAGATTTAGCTATTTTCTCTCTTCATTTAGCAGGGATTTCA
TCAATTTTAGGAGCTGTAAATTTTATTACTACAGTAATTAATATACGATCTCCTGG
AATTACTCTAGATCGAATACCTTTATTTGTTTGATCTGTAGTAATTACAGCTGTAT
TATTATTATTATCTTTACCTGTATTAGCAGGAGCAATTACAATATTATTAACTGAT
CGAAATTTAAATACATCATTCTTCGACCCTGCTGGAGGTGGAGACCC

VBDF 10-05 is a mosquito specimen that was identified as *Anopheles squamosus* using morphological identification.

ATAGTAGGTACTTCTTTAAGTATTCTAATTCGAGCTGAATTAGGACATCCAGGAG
CATTTATTGGAGATGATCAAATTTATAATGTAATCGTAACTGCTCACGCTTTTATT

ATAATTTTTTTTATAGTAATACCAATTATAATTGGGGGATTCGGAAATTGATTAGT
TCCTTTAATATTAGGAGCCCCAGATATAGCTTTCCCTCGAATAAATAATATAAGT
TTTTGAATACTTCCTCCATCTTTAACATTACTTATCTCTAGTAGTATAGTAGAAAA
CGGGGCTGGAACAGGATGAACAGTTTATCCCCCTCTTTCTTCTGGAATTGCTCA
CGCAGGAGCTTCAGTAGATTTAGCTATTTTCTCTCTTCATTTAGCAGGGATTTCA
TCAATTTTAGGAGCTGTAAATTTTATTACTACAGTAATTAATATACGATCTCCTGG
AATTACTCTAGATCGAATACCTTTATTTGTTTGATCTGTAGTAATTACAGCTGTAT
TATTATTATTATCTTTACCTGTATTAGCAGGAGCAATTACAATATTATTAACTGAT
CGAAATTTAAATACATCATTCTTCGACCCTGCTGGAGGTGGAGACCC

VBDF 10-06 is a mosquito specimen that was identified as *Anopheles squamosus* using morphological identification.

ATAGTAGGTACTTCTTTAAGTATTCTAATTCGAGCTGAATTAGGACATCCAGGAG
CATTTATTGGAGATGATCAAATTTATAATGTAATCGTAACTGCTCACGCTTTTATT
ATAATTTTTTTTATAGTAATACCAATTATAATTGGGGGATTCGGAAATTGATTAGT
TCCTTTAATATTAGGAGCCCCAGATATAGCTTTCCCTCGAATAAATAATATAAGT
TTTTGAATACTTCCTCCATCTTTAACATTACTTATCTCTAGTAGTATAGTAGAAAA
CGGGGCTGGAACAGGATGAACAGTTTATCCCCCTCTTTCTTCTGGAATTGCTCA
CGCAGGAGCTTCAGTAGATTTAGCTATTTTCTCTCTTCATTTAGCAGGGATTTCA
TCAATTTTAGGAGCTGTAAATTTTATTACTACAGTAATTAATATACGATCTCCTGG
AATTACTCTAGATCGAATACCTTTATTTGTTTGATCTGTAGTAATTACAGCTGTAT
TATTATTATTATCTTTACCTGTATTAGCAGGAGCAATTACAATATTATTAACTGAT
CGAAATTTAAATACATCATTCTTCGACCCTGCTGGAGGTGGAGACCC

VBDF 10-07 is a mosquito specimen that was identified as *Anopheles squamosus* using morphological identification.

ATAGTAGGTACTTCTTTAAGTATTCTAATTCGAGCTGAATTAGGACATCCAGGAG
CATTTATTGGAGATGATCAAATTTATAATGTAATCGTAACTGCTCACGCTTTTATT
ATAATTTTTTTTATAGTAATACCAATTATAATTGGGGGATTCGGAAATTGATTAGT
TCCTTTAATATTAGGAGCCCCAGATATAGCTTTCCCTCGAATAAATAATATAAGT
TTTTGAATACTTCCTCCATCTTTAACATTACTTATCTCTAGTAGTATAGTAGAAAA
CGGGGCTGGAACAGGATGAACAGTTTATCCCCCTCTTTCTTCTGGAATTGCTCA
CGCAGGAGCTTCAGTAGATTTAGCTATTTTCTCTCTTCATTTAGCAGGGATTTCA
TCAATTTTAGGAGCTGTAAATTTTATTACTACAGTAATTAATATACGATCTCCTGG
AATTACTCTAGATCGAATACCTTTATTTGTTTGATCTGTAGTAATTACAGCTGTAT
TATTATTATTATCTTTACCTGTATTAGCAGGAGCAATTACAATATTATTAACTGAT
CGAAATTTAAATACATCATTCTTCGACCCTGCTGGAGGTGGAGACCC

VBDF 10-09 is a mosquito specimen that was identified as *Anopheles squamosus* using morphological identification.

GAATGATGTATTTAAGTTTCGATCAGTTAATAATATTGTAATTGCTCCTGCTAATA
CAGGTAAAGATAATAATAATAATACAGCTGTAATTACTACAGATCAAACAAATAA
AGGTATTCGATCTAGAGTAATTCCAGGAGATCGTATATTAATTACTGTAGTAATA
AAATTTACAGCTCCTAAAATTGATGAAATCCCTGCTAAATGAAGAGAGAAAATAG
CTAAATCTACTGAAGCTCCTGCGTGAGCAATTCCAGAAGAAAGAGGGGGATAAA
CTGTTCATCCTGTTCCAGCCCCGTTTTCTACTATAGTACTAGAGATAAGTAATGT

TAAAGATGGAGGAAGTATTCAAAAACTTATATTATTTATTCGAGGGAAAGCTATA
TCTGGGGCTCCTAATATTAAAGGAACTAATCAATTTCCGAATCCCCCAATTATAA
TTGGCATTACTATAAAAAAAATTATAATAAAAGCGTGAGCAGTTACGATTACATT
ATAAATTTGGTCATTTCCAATAAATGCTCCTGGATGTCCTAATTCAGCTCGAATT
A

VBDF 10-10 is a mosquito specimen that was identified as *Anopheles squamosus* using morphological identification.

ATCAGTTAATAATATTGTAATTGCTCCTGCTAATACAGGTAAAGATAATAATAATA
ATACAGCTGTAATTACTACAGATCAAACAAATAAAGGTATTCGATCTAGAGTAAT
TCCAGGAGATCGTATATTAATTACTGTAGTAATAAAATTTACAGCTCCTAAAATT
GATGAAATCCCTGCTAAATGAAGAGAGAAAATAGCTAAATCTACTGAAGCTCCT
GCGTGAGCAATTCCAGAAGAAAGAGGGGGATAAACTGTTCATCCTGTTCCAGC
CCCGTTTTCTACTATACTACTAGAGATAAGTAATGTTAAAGATGGAGGAAGTATT
CAAAAACTTATWTTATTTATTCGAGGGAAAGCTATATCTGGGGCTCCTAATATTA
AAGGAACTAATCAATTTCCGAATCCCCCAATTATAATTGGTATTACTATAAAAAAA
ATTATAATAAAAGCGTGAGCAGTTACGATTACATTATAAATTTGATCATCTCCAAT
AAATGCTCCTGGAGGTCCTAATTCASCTCGAATTAGAATACTTAAAGAAGTACCT
ACTATTCCAGCTCAAGCACCG

VBDF 10-11 is a mosquito specimen that was identified as *Anopheles squamosus* using morphological identification.

AATTTAAATTTCGATCAGTTAATAATATTGTAATTGCTCCTGCTAATACAGGTAAA
GATAATAATAATAATACAGCTGTAATTACTACAGATCAAACAAATAAAGGTATTC
GATCTAGAGTAATTCCAGGAGATCGTATATTAATTACTGTAGTAATAAAATTTAC
AGCTCCTAAAATTGATGAAATCCCTGCTAAATGAAGAGAGAAAATAGCTAAATCT
ACTGAAGCTCCTGCGTGAGCAATTCCAGAAGAAAGAGGGGGATAAACTGTTCA
TCCTGTTCCAGCCCCGTTTTCTACTATACTACTAGAGATAAGTAATGTTAAAGAT
GGAGGAAGTATTCAAAAACTTATATTATTTATTCGAGGGAAAGCTATATCTGGGG
CTCCTAATATTAAAGGAACTAATCAATTTCCGAATCCCCCAATTATAATTGGTATT
ACTATAAAAAAAATTATAATAAAAGCGTGAGCAGTTACGATTACATTATAAATTTG
ATCATCTCCAATAAATGCTCCTGGATGTCCTAATTCAGCTCGAATTAGAATACTT
AAAGAAGTACCTACTATTCCAGCTCAAGCACCGAAAATAAAGTATAAAGT

VBDF 10-12 is a mosquito specimen that was identified as *Anopheles squamosus* using morphological identification.

TATAAAATAGGGTCTCCACCTCCAGCAGGGTCGAAGAATGATGTATTTAAATTTC
GATCAGTTAATAATATTGTAATTGCTCCTGCTAATACAGGTAAAGATAATAATAAT
AATACAGCTGTAATTACTACAGATCAAACAAATAAAGGTATTCGATCTAGAGTAA
TTCCAGGAGATCGTATATTAATTACTGTAGTAATAAAATTTACAGCTCCTAAAATT
GATGAAATCCCTGCTAAATGAAGAGAGAAAATAGCTAAATCTACTGAAGCTCCT
GCGTGAGCAATTCCAGAAGAAAGAGGGGGATAAACTGTTCATCCTGTTCCAGC
CCCGTTTTCTACTATACTACTAGAGATAAGTAATGTTAAAGATGGAGGAAGTATT
CAAAAACTTATATTATTTATTCGAGGGAAAGCTATATCTGGGGCTCCTAATATTA
AAGGAACTAGTCAATTTCCGAATCCCCCAATTATAATTGGTATTACTATAAAAAA
AATTATAATAAAAGCGTGAGCAGTTACGATTACATTATAAATTTGATCATCTCCAA

TAAATGCTCCTGGATGTCCTAATTCAGCTCGAATTAGAATACTTAAAGAAGTACC
TACTATTCCAG

VBDF 11-22 is a mosquito specimen that was identified as *Anopheles squamosus* using morphological identification.

ATAGTAGGTACTTCTTTAAGTATTCTAATTCGAGCTGAATTAGGACATCCAGGAG
CATTTATTGGAGATGATCAAATTTATAATGTAATCGTAACTGCTCACGCTTTTATT
ATAATTTTTTTTATAGTAATACCAATTATAATTGGGGGATTCGGAAATTGATTAGT
TCCTTTAATATTAGGAGCCCCAGATATAGCTTTCCCTCGAATAAATAATATAAGT
TTTTGAATACTTCCTCCATCTTTAACATTACTTATCTCTAGTAGTATAGTAGAAAA
CGGGGCTGGAACAGGATGAACAGTTTATCCCCCTCTTTCTTCTGGAATTGCTCA
CGCAGGAGCTTCAGTAGATTTAGCTATTTTCTCTCTTCATTTAGCAGGGATTTCA
TCAATTTTAGGAGCTGTAAATTTTATTACTACAGTAATTAATATACGATCTCCTGG
AATTACTCTAGATCGAATACCTTTATTTGTTTGATCTGTAGTAATTACAGCTGTAT
TATTATTATTATCTTTACCTGTATTAGCAGGAGCAATTACAATATTATTAACTGAT
CGAAATTTAA

VBDF 11-23 is a mosquito specimen that was identified as *Anopheles squamosus* using morphological identification.

ATAGGGTCTCCACCTCCAGCAGGGTCGAAGAATGATGTATTTAAATTTCGATCA
GTTAATAATATTGTAATTGCTCCTGCTAATACAGGTAAAGATAATAATAATAATAC
AGCTGTAATTACTACAGATCAAACAAATAAAGGTATTCGATCTAGAGTAATTCCA
GGAGATCGTATATTAATTACTGTAGTAATAAAATTTACAGCTCCTAAAATTGATG
AAATCCCTGCTAAATGAAGAGAGAAAATAGCTAAATCTACTGAAGCTCCTGCGT
GAGCAATTCCAGAAGAAAGAGGGGGATAAACTGTTCATCCTGTTCCAGCCCCG
TTTTCTACTATACTACTAGAGATAAGTAATGTTAAAGATGGAGGAAGTATTCAAA
AACTTATATTATTTATTCGAGGGAAAGCTATATCTGGGGCTCCTAATATTAAAGG
AACTAATCAATTTCCGAATCCCCCAATTATAATTGGTATTACTATAAAAAAAATTA
TAATAAAAGCGTGAGCAGTTACGATTACATTATAAATTTGATCATCTCCAATAAAT
GCTCCTGGATGTCCTAATTCAGCTCGAATTAGAATACTTAAAGAAGTACCTACTA
TTCCAGCTCAAGCACCGAAA

VBDF 11-24 is a mosquito specimen that was identified as *Anopheles squamosus* using morphological identification.

TAATAATAATAATACAGCTGTAATTACTACAGATCAAACAAATAAAGGTATTCGAT
CTAGAGTAATTCCAGGAGATCGTATATTAATTACTGTTGTAATAAAATTTACAGC
TCCTAAAATTGATCGAAATCCCTGCTAAATGAAGAGAGAAAATAGCTAAATCTAC
TGAAGCTCCTGCATGAGCAATTCCAGAAGAGAAGAGGGGGATAAACTGTTCAT
CCTGTTCCTGCCCCGTTTTCTATTATGCTACTAGACATAAGTAATGTTAAAGAGG
GAGGAAGTATTCAAAAACTTATATTATTTATTCGAGGGAAAGCTATATCTGGGGC
TCCTAATATTAAAGGAACTAATCAATTTCCCAATCCCCCAATTATAATTGGATTAC
TATAAAGAAAATTATAATAAATGCGTGAGCAGTTACGATTACATTATAAATTTGAT
CATCTCCAATAAATGCTCCTGGATGTCCTAATTCAGCTCGAATTAGAATACTTAA
AGAAGTACCTACTATTCCAGCTCAAGCACCG

VBDF 11-25 is a mosquito specimen that was identified as *Anopheles squamosus* using morphological identification.

ATAGGGTCTCCACCTCCAGCAGGGTCGAAGAATGATGTATTTAAATTTCGATCA
GTTAATAATATTGTAATTGCTCCTGCTAATACAGGTAAAGATAATAATAATAATAC
AGCTGTAATTACTACAGATCAAACAAATAAAGGTATTCGATCTAGAGTAATTCCA
GGAGATCGTATATTAATTACTGTAGTAATAAAATTTACAGCTCCTAAAATTGATG
AAATCCCTGCTAAATGAAGAGAGAAAATAGCTAAATCTACTGAAGCTCCTGCGT
GAGCAATTCCAGAAGAAAGAGGGGGATAAACTGTTCATCCTGTTCCAGCCCCG
TTTTCTACTATACTACTAGAGATAAGTAATGTTAAAGATGGAGGAAGTATTCAAA
AACTTATATTATTTATTCGAGGGAAAGCTATATCTGGGGCTCCTAATATTAAAGG
AACTAATCAATTTCCGAATCCCCCAATTATAATTGGTATTACTATAAAAAAAATTA
TAATAAAAGCGTGAGCAGTTACGATTACATTATAAATTTGATCATCTCCAATAAAT
GCTCCTGGATGTCCTAATTCAGCTCGAATTAGAATACTTAAAGAAGTACCTACTA
TTCCAGCTCAAGCACCGAAA

VBDF 11-26 is a mosquito specimen that was identified as *Anopheles squamosus* using morphological identification.

TCGAAGAATGATGTATTTAAATTTCGATCAGTTAATAATATTGTAATTGCTCCTGC
TAATACAGGTAAAGATAATAATAATAATACAGCTGTAATTACTACAGATCAAACA
AATAAAGGTATTCGATCTAGAGTAATTCCAGGAGATCGTATATTAATTACTGTAG
TAATAAAATTTACAGCTCCTAAAATTGATGAAATCCCTGCTAAATGAAGAGAGAA
AATAGCTAAATCTACTGAAGCTCCTGCGTGAGCAATTCCAGAAGAAAGAGGGG
GATAAACTGTTCATCCTGTTCCAGCCCCGTTTTCTACTATACTACTAGAGATAAG
TAATGTTAAAGATGGAGGAAGTATTCAAAAACTTATATTATTTATTCGAGGGAAA
GCTATATCTGGGGCTCCTAATATTAAAGGAACTAATCAATTTCCGAATCCCCCAA
TTATAATTGGTATTACTATAAAAAAAATTATAATAAAAGCGTGAGCAGTTACGATT
ACATTATAAATTTGATCATCTCCAATAAATGCTCCTGGATGTCCTAATTCAGCTC
GAATTAGAATACTTAAAGAAGTACCTACTAT

5.2. Meteorological data of the Bloemfontein area

Over the last 15 years the summer temperatures of the Bloemfontein City centre stayed relatively constant. A steady increase was seen from 2002 and the maximum summer temperatures are almost 3°C warmer from 2015-2017. Temperatures for 2017 do not include December, which is one of the warmest months of the year with temperatures reaching well beyond 30°C. The minimum temperature for summer has stayed very constant throughout the 15 years that it has been measured. On average Bloemfontein city centre has a minimum summer temperature of 13,03°C and a maximum summer temperature of 28,85°C. 2003, 2004, 2005, 2007, 2013, 2014, 2015, 2016 and 2017 all had above average temperatures with 2015 warmest and 2006 being the coldest to date. The summer temperatures for the Bloemfontein City centre since 2002 are shown in Figure 5.2.1.

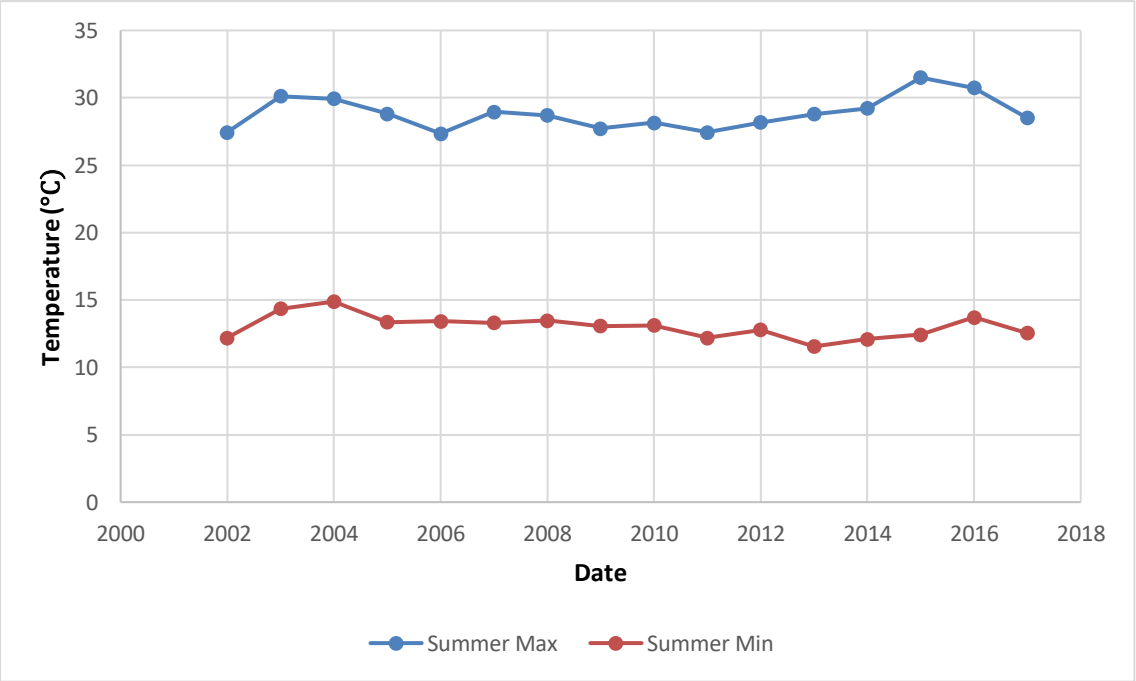


Figure 5.2.1: Summer temperatures of the Bloemfontein City centre from 2002 – 2017.

Winter temperatures have been rising over the last 15 years have slowly increased. The average maximum temperature for the Bloemfontein city centre is 21,75°C and the minimum temperature was 5,01°C. The maximum winter temperature has increased by 2°C on average since 2002. The years of 2013, 2014, 2015, 2016 and

2017 had above average temperatures. 2014 had the highest recorded maximum temperature was in 2014. The lowest maximum temperatures were recorded in 2006 and 2011. The highest minimum winter temperature was recorded in 2008 and the lowest minimum winter temperature was recorded in 2014. The winter temperatures for the Bloemfontein City centre from 2002 is shown in Figure 5.2.2.

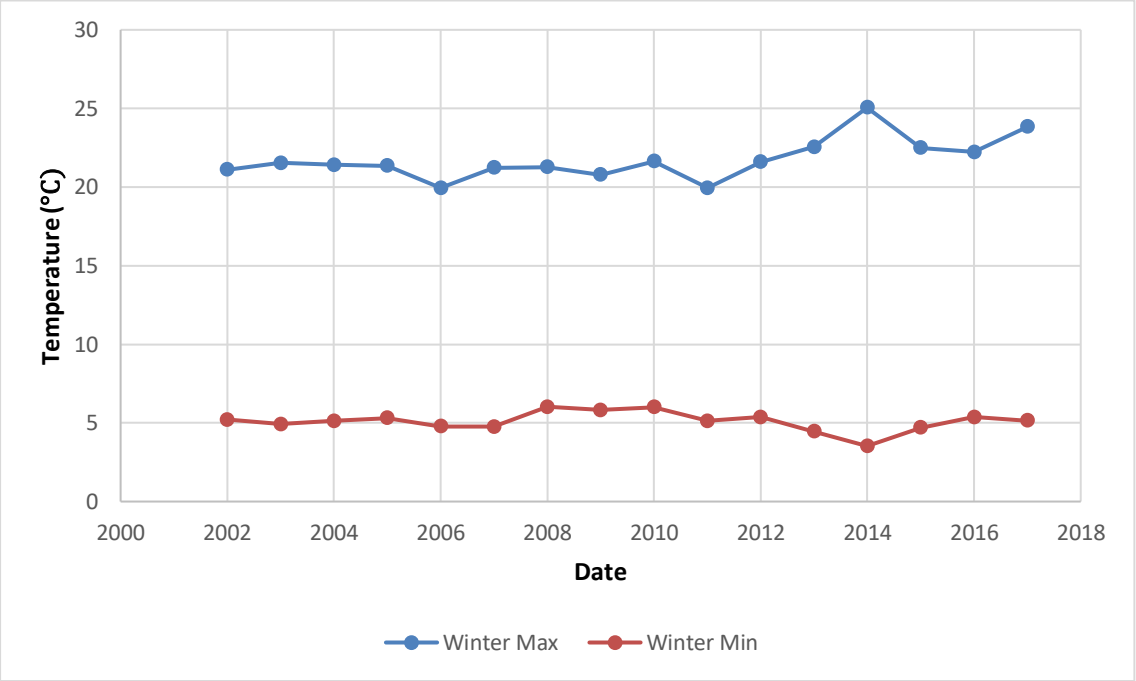


Figure 5.2.2: Winter temperatures of the Bloemfontein City centre from 2002 – 2017.

The rainfall in the Bloemfontein City centre was quite variable since 2002. Over the last 15 years the total rainfall for the Bloemfontein City centre was 7993,8 mm and the average rainfall is 532,92 mm. The highest recorded rainfall was in 2006, 2010 and 2011 reaching total rainfall of over 700mm each year. The lowest rainfall was recorded in 2015 with only 287,2 mm. The rainfall for 2016 and 2017 show similar trends as in 2004/2005 and 2012/2013. With an increase in rainfall expected. The year of 2017 has shown a below average rainfall. The average rainfall for the Bloemfontein City centre from 2002 to 2017 is shown in Figure 5.2.3.

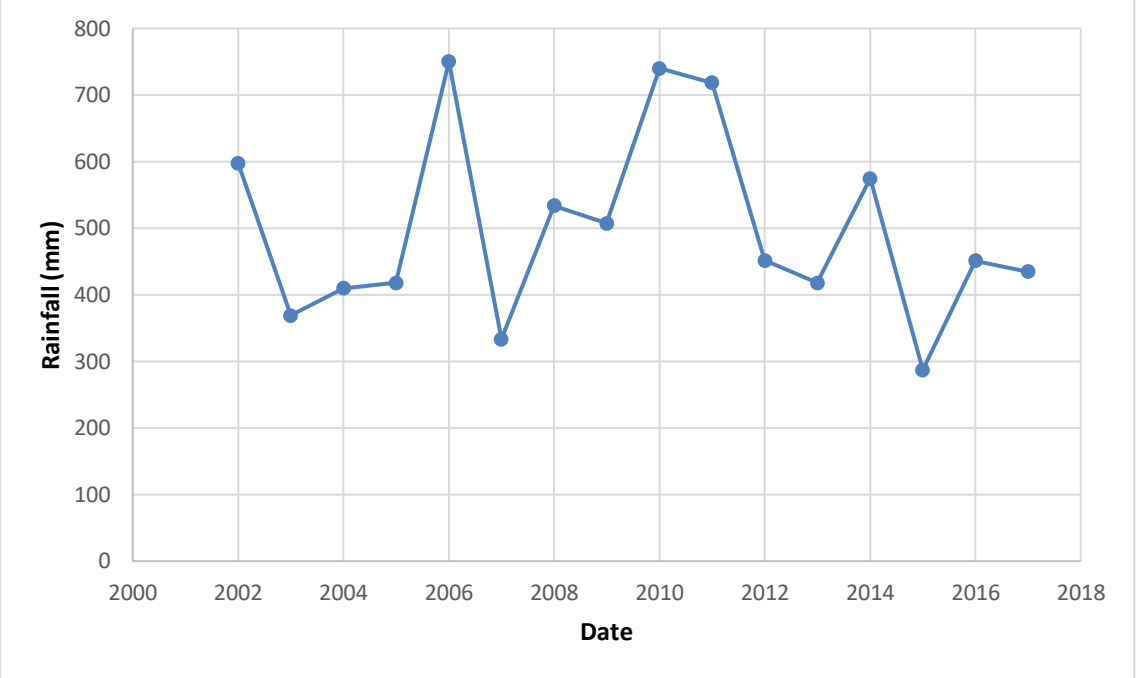


Figure 5.2.3: Average rainfall of the Bloemfontein City centre from 2002 – 2017.

The summer temperatures of the Glen Agricultural station close to the Free State National Botanical Gardens. The maximum summer temperatures have shown a slight increase over the last 13 years. The average maximum summer temperature for the past 13 years is 29,96°C The maximum temperatures have stayed relatively constant with an average increase of around 1°C. The minimum summer temperatures have also been constant with no marked change over time. The average minimum winter temperature is 11,98°C. The highest maximum summer temperature was in 2015 and the lowest maximum summer temperature was in 2006. The highest minimum summer temperature was recorded in 2016 and the lowest minimum summer temperature was recorded in 2015. The years of 2012, 2013, 2014, 2015 and 2016 all shown above average summer temperatures. The graph does not have data including December 2017, which is one of the warmest months. It is predicted that 2017 is on average also warmer than average. The weather station also shows higher temperatures as compared to the Bloemfontein City centre. The summer temperatures of the Glen weather station from 2004 to 2017 are shown in Figure 5.2.4.

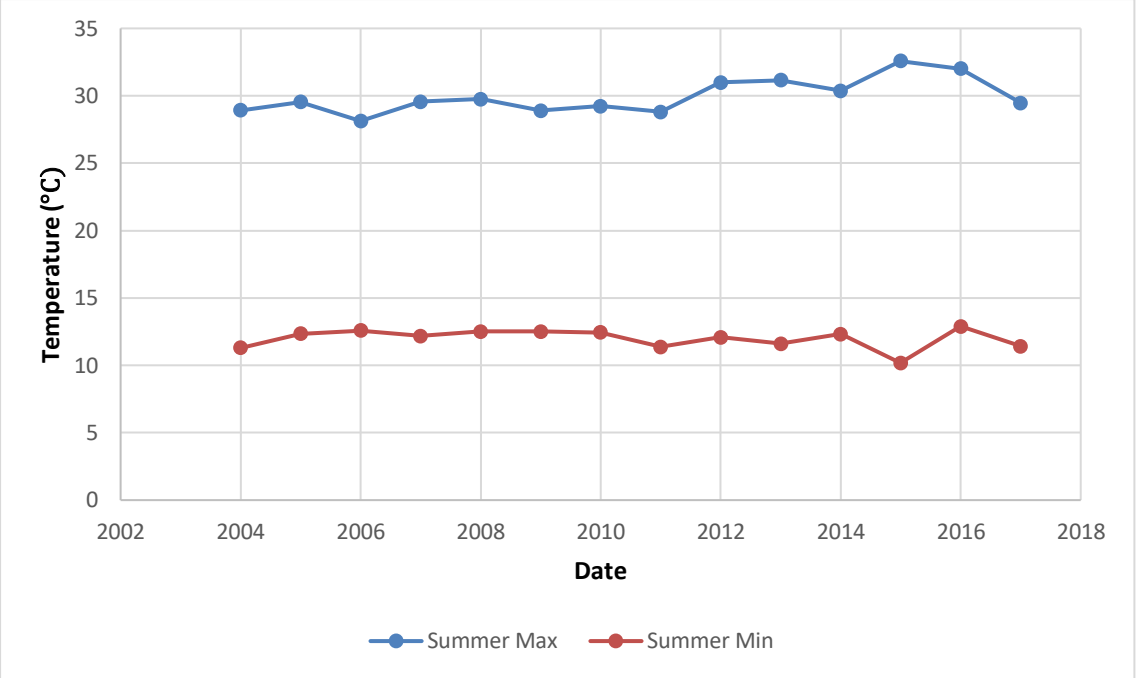


Figure 5.2.4: Summer temperatures of the Glen Weather Station from 2004 – 2017.

Winter maximum temperatures have shown a slight increase over the past 13 years. The maximum winter temperature is on average 4°C higher in 2017 than it was in 2004. The average maximum winter temperature is 22,58°C. In 2005, 2010, 2012, 2013, 2014, 2015, 2016 and 2017 had above average maximum winter temperatures. The highest maximum temperature was recorded in 2017. The minimum winter temperatures have stayed constant with exception to 2007, 2014 and 2017 being colder than the other years. The average minimum winter temperature is 3,85°C. The highest minimum winter temperature was recorded in 2010and the lowest minimum winter temperature was observed in 2007. On the graph it appears that 2004 has the lowest minimum winter temperature, but data was only available for one month and it is therefore not a true representation of the winter temperature for that year. The Winter temperatures of the Glen Weather Station from 2004-2017 is shown in Figure 5.2.5.

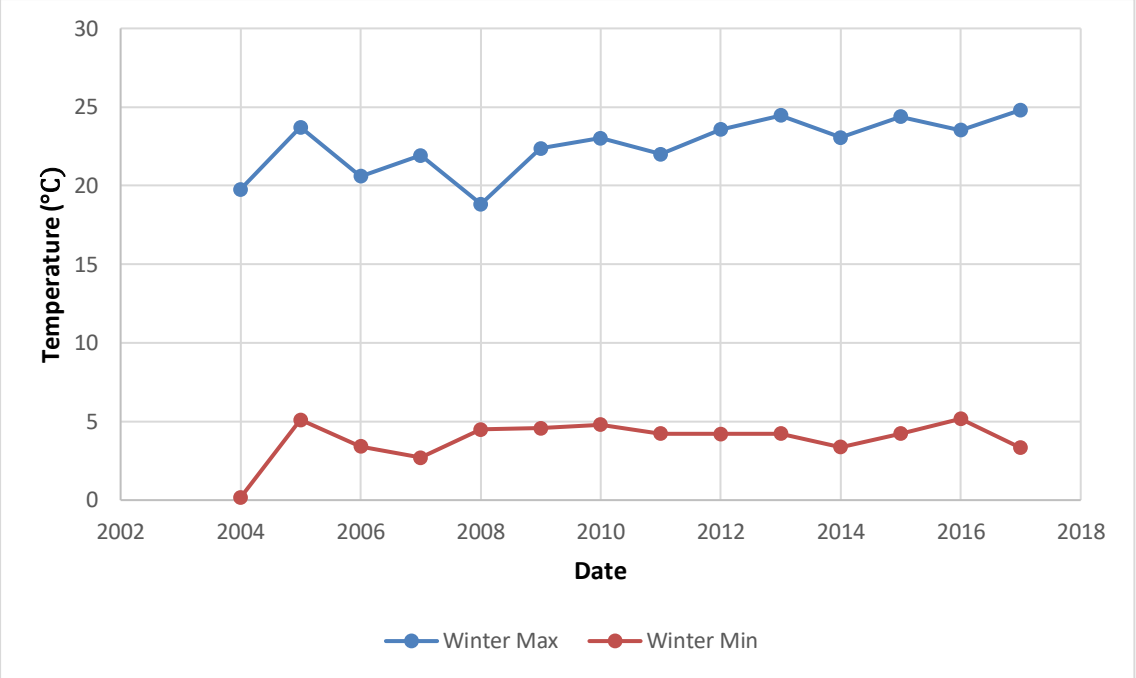


Figure 5.2.5: Winter temperatures of the Glen Weather Station from 2004 – 2017.

Weather data is only available from June of 2004 so for the calculation of averages and total rainfall it was not considered to provide accurate results. The rainfall in the area is variable and does not follow a specific pattern. The total rainfall from 2005 to November of 217 is 6349,2 mm with an average rainfall of 488,4 mm. In 2014 the rainfall was well beyond 700 mm and showed the highest recorded rainfall in 12 years. The lowest rainfall was documented in 2017 with a drought known to occur after good rainfall in 2016. The average rainfall for the Glen Weather Station from 2004 to 2017 is shown in Figure 5.2.6.

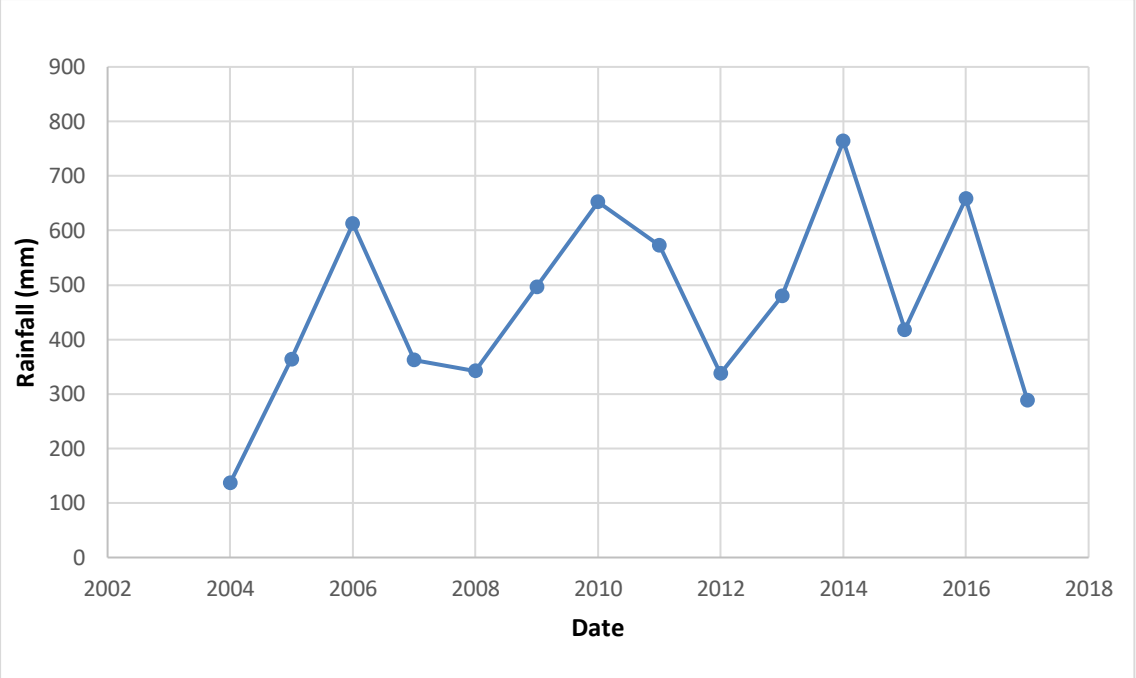


Figure 5.2.6: Average rainfall of the Glen Weather Station from 2004 – 2017.

The Bloemfontein West weather station is the closest weather station to the Krugersdrift Dam. The maximum summer temperature has shown a slight increase since 2002. Maximum summer temperatures are on average 2°C higher than in 2002.The average maximum summer temperature is 28,81°C. Above average maximum summer temperatures have been recorded in 2003, 2008, 2013, 2014, 2015 and 2016. Temperature data was not available for December 2017 and it is one of the warmest months of the year. If this month is to be included it is predicted that 2017 will also show above average temperatures. The highest maximum summer temperature was recorded in 2016 and the lowest minimum summer temperature was recorded in 2011. The minimum summer temperatures have shown an increase since 2002, but showed a decrease in 2007, 2011 and 2017. Temperatures seem to follow a pattern of increasing and decreasing slightly. The average minimum summer temperature is 11,50°C. The highest minimum summer temperature was recorded in 2016 and the lowest minimum summer temperature was recorded in 2011. The summer temperatures for Bloemfontein West weather station for 2002 to 2017 are shown in Figure 5.2.7.

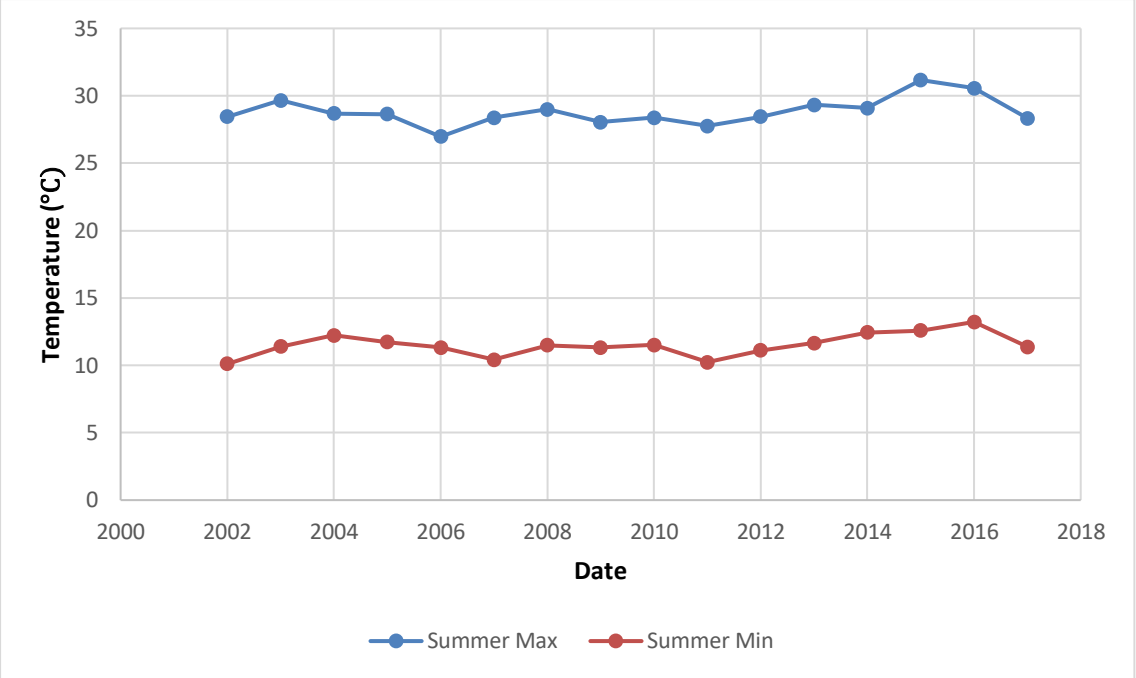


Figure 5.2.7: Summer temperatures of the Bloemfontein West weather station 2002– 2017.

The winter temperatures showed a slight increase since 2002. The temperature on average has increased by approximately 1°C. The average maximum winter temperature is 21,80°C. In 2002, 2003, 2010,2012, 2013, 2014, 2015, 2016 and 2017 above average maximum winter temperatures have been recorded. The highest maximum winter temperature was recorded in 2017 and the lowest maximum winter temperature was recorded in 2006. The minimum winter temperatures showed a steady decrease until 2007 and then slowly increased until 2016. The average minimum winter temperature is 3,14°C. The highest minimum winter temperature was recorded in 2016 and the lowest minimum winter temperature was recorded in 2007. The winter temperatures of the Bloemfontein West weather station 2002-2017 are shown in Figure 5.2.8.

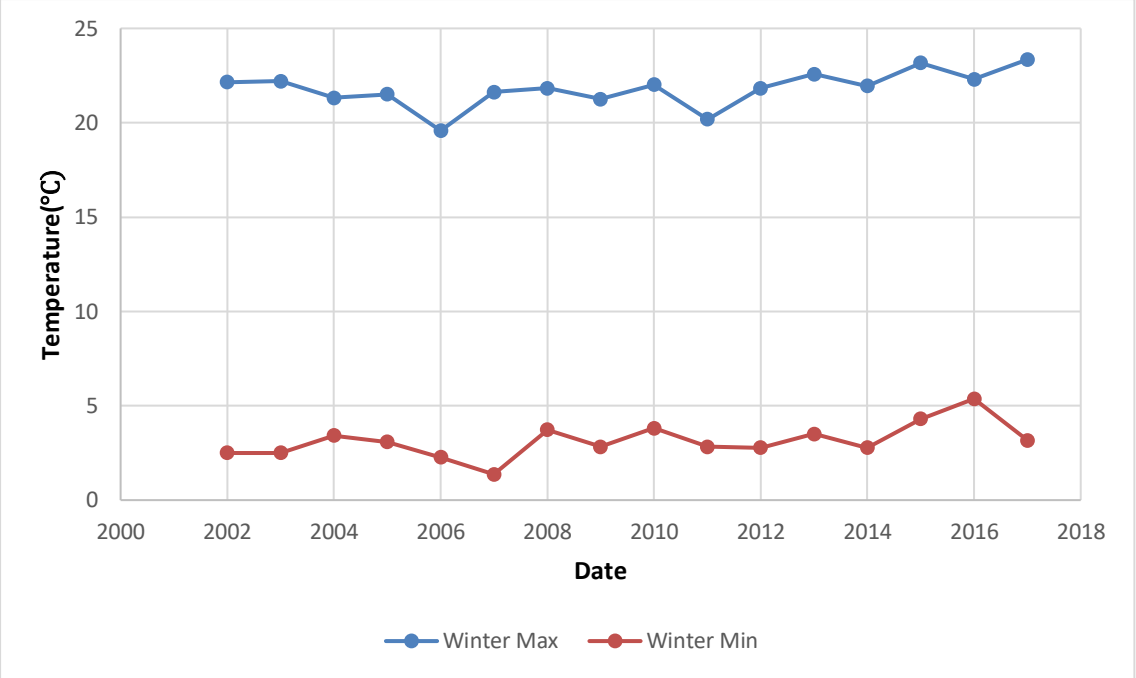


Figure 5.2.8: Winter temperatures of the Bloemfontein West weather station 2002– 2017.

The rainfall in the area is variable, showing an average decrease of rainfall over time. The total rainfall for the area since 2002 is 7951,7 mm and the average rainfall is 496,98 mm. 2010 and 2011 had rainfall of above 700 mm and 2006 had a very high rainfall of well over 800 mm. The lowest rainfall was recorded in 2005. The rainfall does not show any specific pattern. The average rainfall for the Bloemfontein West weather station is shown in Figure 5.2.9.

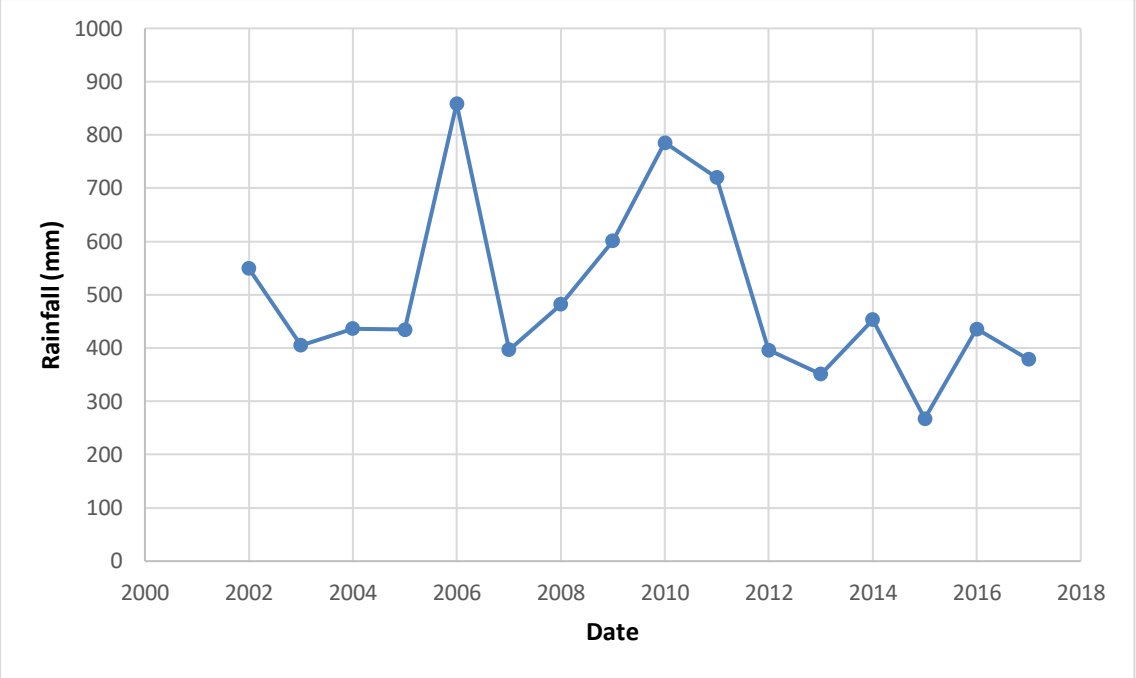


Figure 5.2.9: Average rainfall of the Bloemfontein West weather station 2002– 2017.

The temperature has increased in the Bloemfontein area with above average temperatures recorded since 2013. The summer temperatures for 2017 would also have been higher if data was included for December 2017, that is one of the warmest months of the year. The highest maximum summer temperatures were experienced in 2015 and the lowest maximum summer temperature was recorded in 2006. The average maximum summer temperature over all 15 years is 29,19 and there has been an increase of around 2°C in the maximum summer temperatures. The minimum summer temperatures have been more stable over the last 15 years with above average temperatures only seen in 2003, 2004, 2016 and 2017. The average minimum summer temperature is 12,25°C. The highest minimum summer temperature was in 2016 and the lowest minimum summer temperature was recorded in 2011. The combined average summer temperatures of the three Bloemfontein weather stations from 2002 – 2017 is shown in Figure 5.2.10.

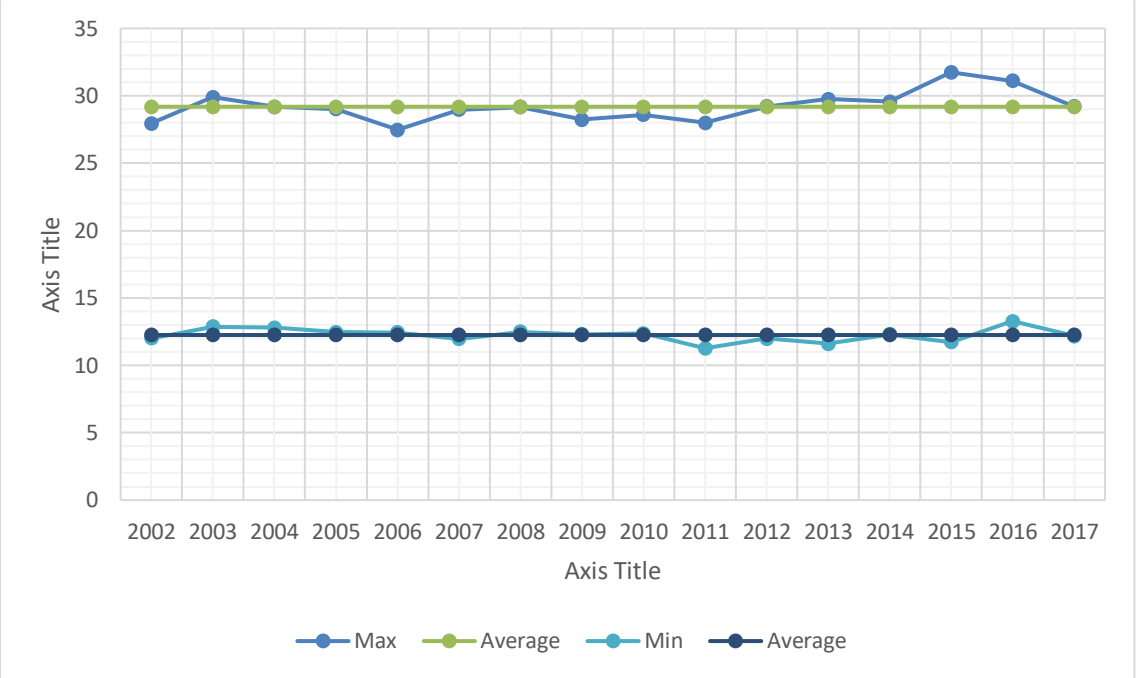


Figure 5.2.10: Summer temperatures of the Bloemfontein Area 2002– 2017.

The maximum winter temperatures of the Bloemfontein area have increased since 2002 with above average temperatures recorded from 2005, 2013, 2014, 2015 and 2016. 2017 had an average maximum winter temperature that was only slightly below average. The average maximum winter temperature for Bloemfontein is 21,89°C. The highest recorded maximum winter temperature was recorded in 2014 and the lowest recorded maximum winter temperature was in 2006. The minimum temperature seems to show pronounced fluctuations around the average. The average minimum winter temperature is 4,04°C. Since 2015 temperatures have been above average and in 2017 temperatures were average. The lowest minimum winter temperatures were recorded in 2004 and the highest minimum winter temperatures were recorded in 2016. The average winter temperatures of the Bloemfontein area from 2002-2017 can be seen in Figure 5.2.11.

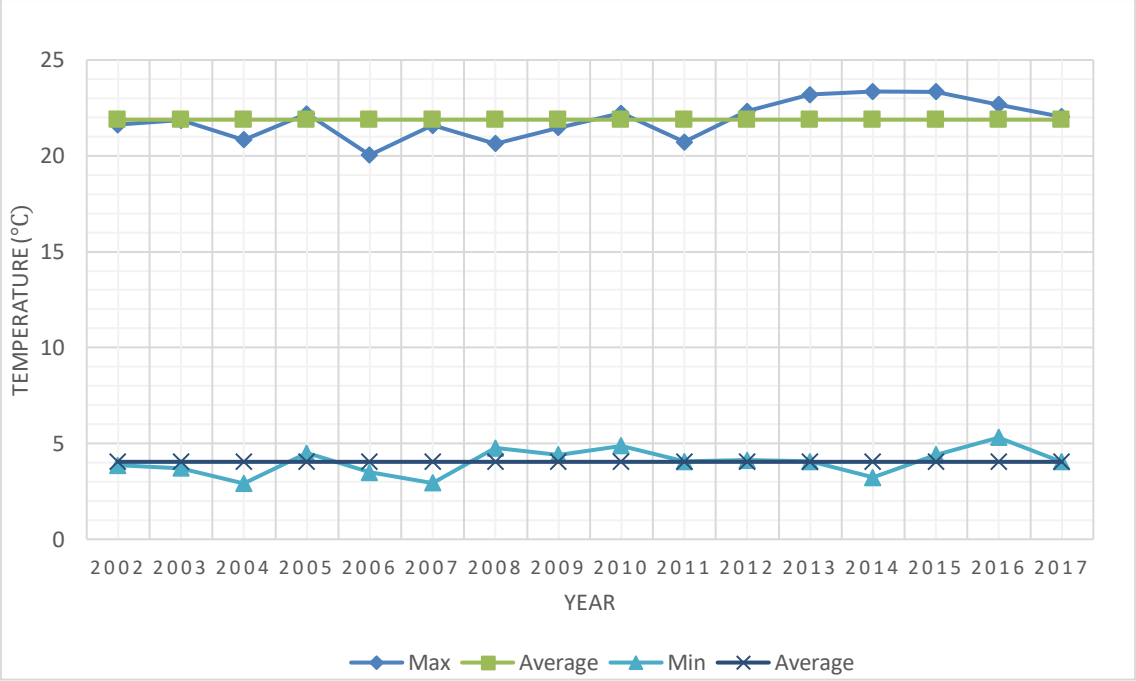


Figure 5.2.11: Winter temperatures of the Bloemfontein Area 2002– 2017.

The rainfall in the Bloemfontein area has showed an overall decrease. The total rainfall for Bloemfontein over the last 15 years is 7892,75 mm with an average rainfall of 493,3 mm. In 2006 and 2010 very good rainfall was recorded with more than 700 mm recorded. In 2015 and 2017 low rainfall was recorded with values under 400 mm recorded. It is known that many areas of South Africa are suffering from drought conditions in 2017 and from the rainfall data it is clear to see Bloemfontein is also affected by this. The average rainfall of the Bloemfontein area from 2002-2017 can be seen in Figure 5.2.12.

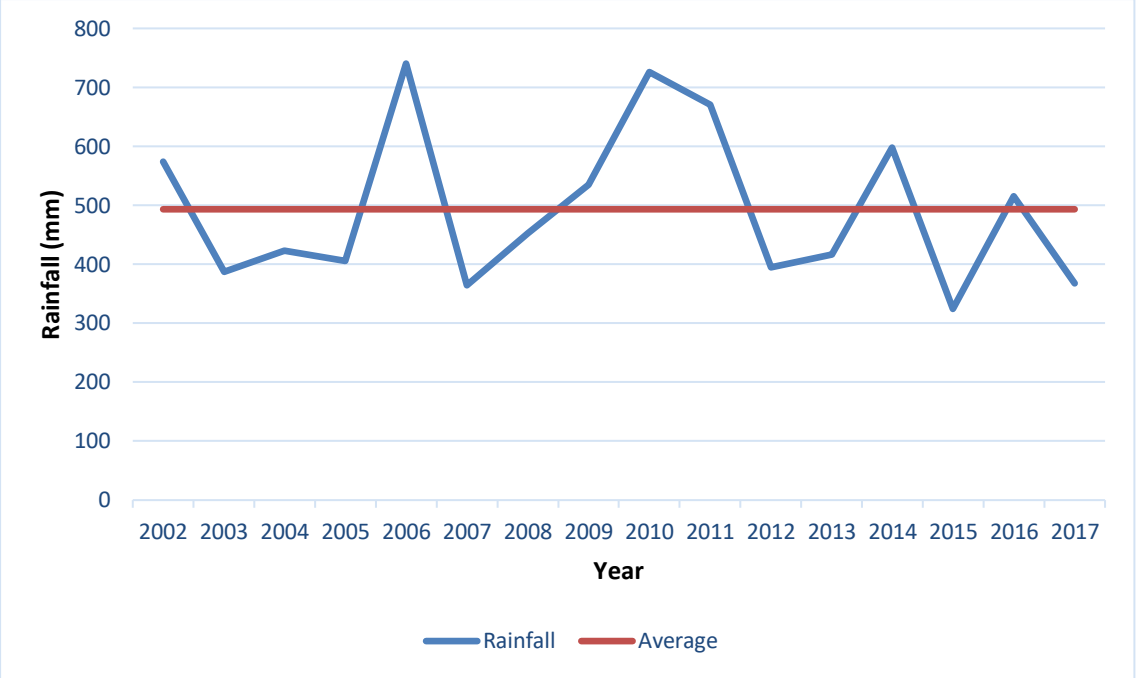


Figure 5.2.12: Winter temperatures of the Bloemfontein Area 2002– 2017.

5.3 Alignments of WNV and WSLV RNA controls to reference sequences from GenBank

In Figure 5.3.1 the sequence alignment of the WNV control and the WNV SA93/01 reference sequence can be seen. The forward primer is given in blue and the reverse primer can be seen in pink.

Figure 5.3.1: Sequence alignment of WNV control and WNV SA93/01 reference sequence.

In Figure 5.3.2 the sequence alignment of the WNV control and the WNV SAH-177 reference sequence can be seen. The forward primer is given in blue and the reverse primer can be seen in pink.

5.4 Media, buffers and solutions used

50X TAE stock pH

1. Tris-Base-242 g.
2. Acetate (100% acetic acid)-57.1 ml.
3. EDTA: 100 ml (0.5M sodium EDTA)
4. Add dH_2O to one liter.

1XTAE (pH 8.0)

1. Dilute 20 ml from 50X stock into 980 ml dH_2O.

1% Agarose gel

1. Weigh one gram of Seakem®LE agarose powder (Lonza, Maine, USA) and mix it with 100 ml of 1XTAE buffer pH 8.
2. Heat mixture in a microwave oven until agarose powder is completely dissolved.

Luria Bertani broth media

1. Mix 10 g Bacto-Tryptone, 5 g Bacto-Yeast extract and 10 g NaCl in 900 ml H_20.
2. Adjust the pH to 7.0 with 10 M NaOH (approximately 200 µl).
3. Adjust volume to 1 liter with H_2O.
4. Sterilize by autoclaving and store at room temperature.

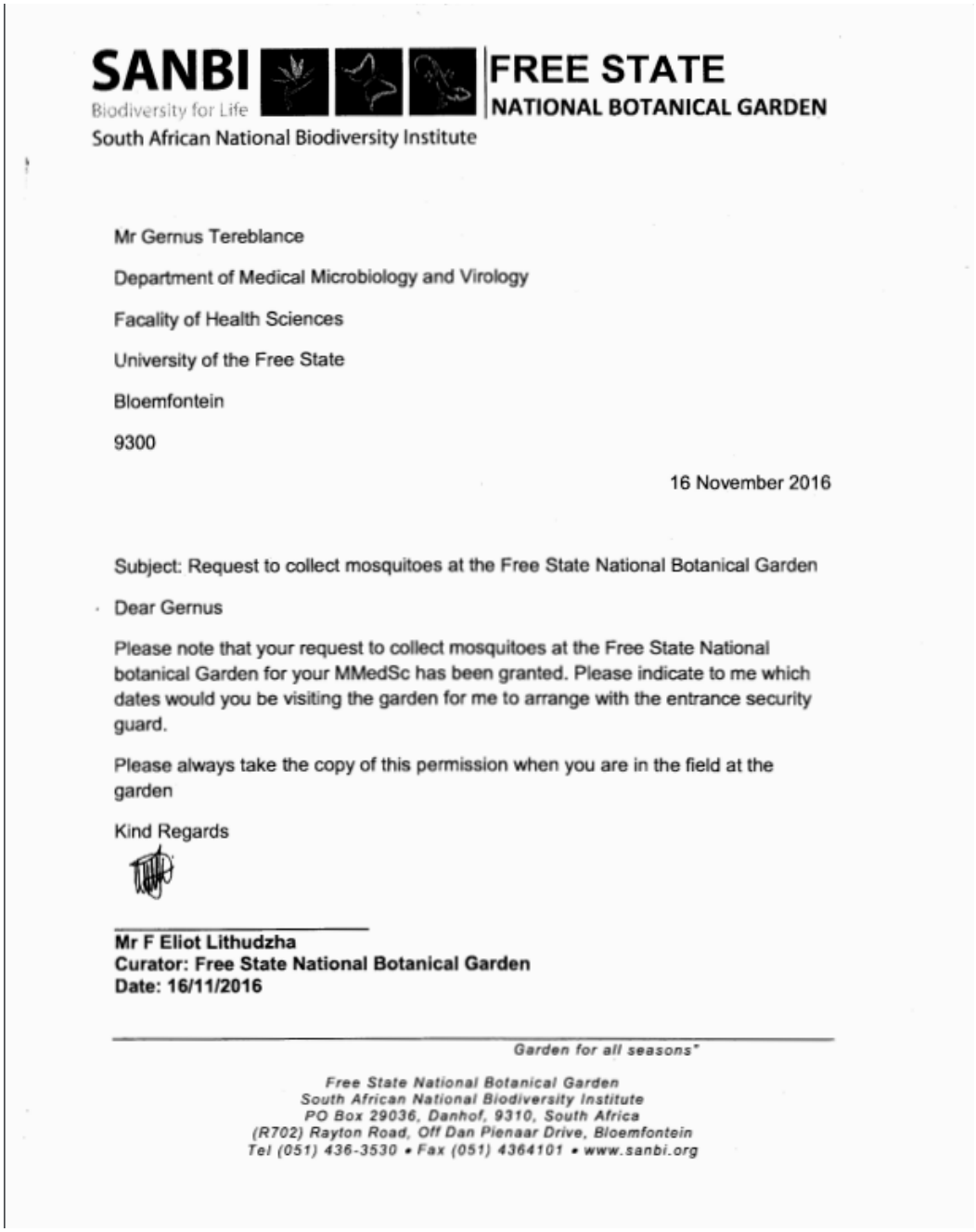
SOC medium

1. Mix 20 g Bacto-Tryptone, 5 g Bacto-Yeast extract, 0.5 g NaCl and 2.5 ml 1 M KCl in 900 ml H_2O.
2. Adjust the pH to 7.0 with 10 M NaOH (approximately 100 µl) and add H_2O to 990 ml.

3. Sterilize by autoclaving and store at room temperature.

4. Before use, add 10ml sterile 1 M $MgCl_2$ and 20 ml sterile 1 M glucose.

5.5. Permissions and permits



SANBI Biodiversity for Life
South African National Biodiversity Institute

FREE STATE
NATIONAL BOTANICAL GARDEN

Mr Gernus Tereblance

Department of Medical Microbiology and Virology

Facality of Health Sciences

University of the Free State

Bloemfontein

9300

16 November 2016

Subject: Request to collect mosquitoes at the Free State National Botanical Garden

Dear Gernus

Please note that your request to collect mosquitoes at the Free State National botanical Garden for your MMedSc has been granted. Please indicate to me which dates would you be visiting the garden for me to arrange with the entrance security guard.

Please always take the copy of this permission when you are in the field at the garden

Kind Regards

Mr F Eliot Lithudzha
Curator: Free State National Botanical Garden
Date: 16/11/2016

Garden for all seasons"

Free State National Botanical Garden
South African National Biodiversity Institute
PO Box 29036, Danhof, 9310, South Africa
(R702) Rayton Road, Off Dan Pienaar Drive, Bloemfontein
Tel (051) 436-3530 • Fax (051) 4364101 • www.sanbi.org

Figure 5.5.1: Permission to collect mosquitoes at the Free State National Botanical Gardens.

OFFICIAL PERMIT

THE DEPARTMENT OF ECONOMIC DEVELOPMENT, TOURISM AND ENVIRONMENTAL AFFAIRS

the detea
the department of economic development, tourism and environmental affairs
FREE STATE PROVINCE

98 ZASTRON STREET
AGRICULTURAL BUILDING
THIRD FLOOR
BLOEMFONTEIN, 9300
FREE STATE
SOUTH AFRICA

PERMIT OFFICE
PRIVATE BAG X20601
BLOEMFONTEIN, 9300
TEL: +27 (0) 51 400 9527/26
FAX: +27 (0) 51 400 9523

PERMIT HOLDER DETAILS

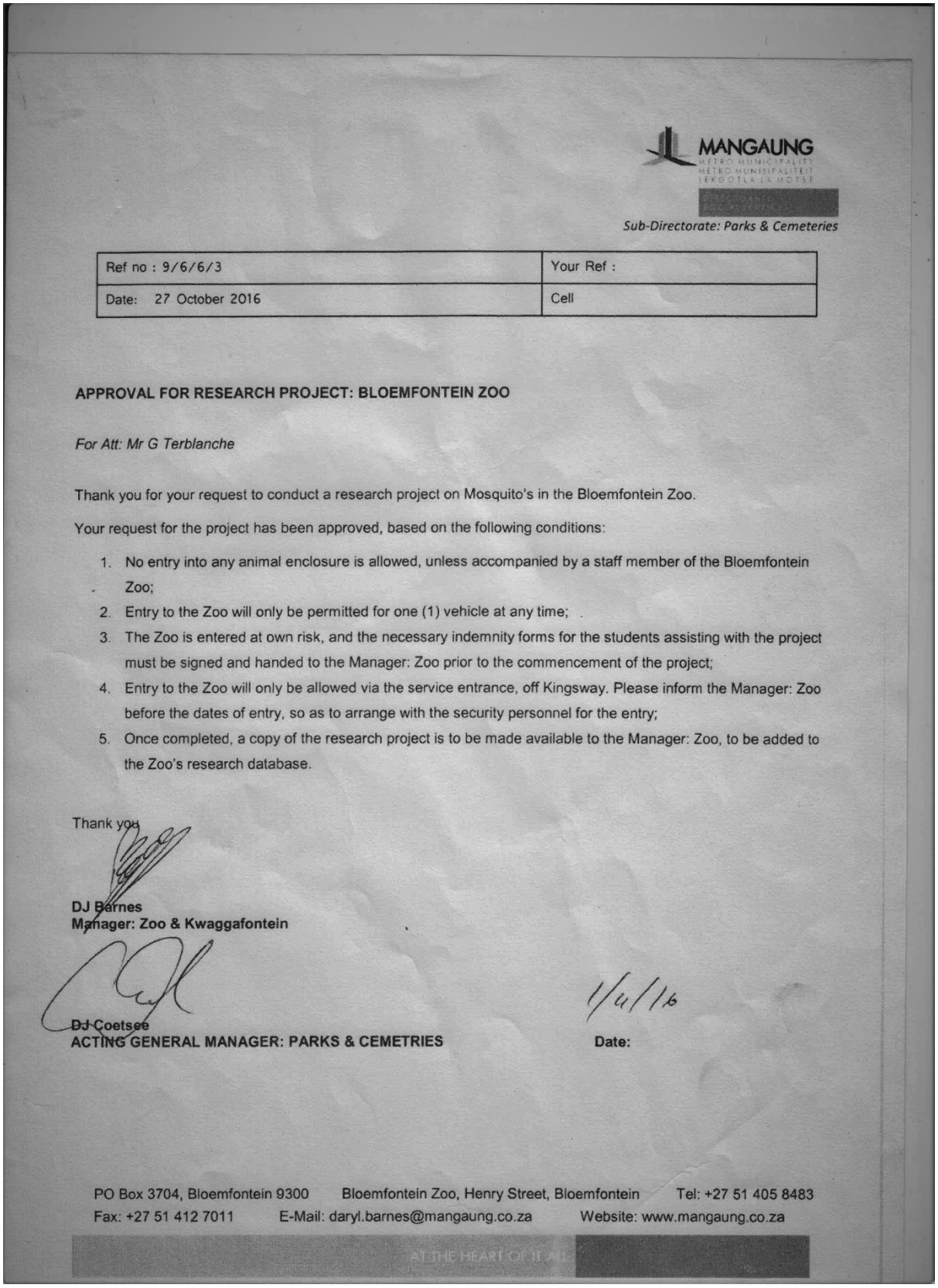
ID NUMBER	9304065089085		
NAME	Gert Ignatius du Preez Terblanche		
PHYSICAL ADDRESS	10 Arndt Street Universitas Bloemfontein 9301	POSTAL ADDRESS	PO Box 102 Hartswater 8570
MOBILE NUMBER:	0790396914		
FAX NUMBER			
EMAIL ADDRESS	gidupterblanche@gmail.com		

THIS PERMIT IS ISSUED IN TERMS OF THE NATIONAL ENVIRONMENTAL MANAGEMENT: PROTECTED AREAS ACT (2003), THE REGUALTIONS FOR THE PROPER ADMINISTRATION OF NATURE RESERVES (2012) AND IN TERMS OF THE FREE STATE NATURE CONSERVATION ORDINANCE (8 OF 1969).
AUTHORISATION IS HEREBY GRANTED TO THE HOLDER OF THIS PERMIT TO;

Conduct research on Mosquitoes at the Soetdoring Nature Reserve in accordance with the project proposal entitled "Identification of arboviruses circulating in mosquitoes populations in the Bloemfontein area: Genus Terblanche" and the special conditions attached hereto.

PERMIT HOLDER'S SIGNATURE	APPROVED ON BEHALF OF THE MEC: DEPARTMENT OF ECONOMIC AND SMALL BUSINESS DEVELOPMENT, TOURISM AND ENVIRONMENTAL AFFAIRS	
EXPIRY DATE 2017/11/30 RETURN PERMIT AFTER EXPIRY	PERMIT NUMBER DH 001/2016	DATE ISSUED 14/11/2016

Figure 5.5.2: Permit to catch mosquitoes at the Soetdoring Nature Reserve (Krugersdrift Dam).



MANGAUNG
METRO MUNICIPALITY
MÉTRO MUNISIPALITEIT
LEKGOTLA LA MOTSE

Sub-Directorate: Parks & Cemeteries

Ref no : 9/6/6/3	Your Ref :
Date: 27 October 2016	Cell

APPROVAL FOR RESEARCH PROJECT: BLOEMFONTEIN ZOO

For Att: Mr G Terblanche

Thank you for your request to conduct a research project on Mosquito's in the Bloemfontein Zoo.

Your request for the project has been approved, based on the following conditions:

1. No entry into any animal enclosure is allowed, unless accompanied by a staff member of the Bloemfontein Zoo;
2. Entry to the Zoo will only be permitted for one (1) vehicle at any time;
3. The Zoo is entered at own risk, and the necessary indemnity forms for the students assisting with the project must be signed and handed to the Manager: Zoo prior to the commencement of the project;
4. Entry to the Zoo will only be allowed via the service entrance, off Kingsway. Please inform the Manager: Zoo before the dates of entry, so as to arrange with the security personnel for the entry;
5. Once completed, a copy of the research project is to be made available to the Manager: Zoo, to be added to the Zoo's research database.

Thank you

DJ Barnes
Manager: Zoo & Kwaggafontein

DJ Coetsee
ACTING GENERAL MANAGER: PARKS & CEMETRIES

1/11/16
Date:

PO Box 3704, Bloemfontein 9300 Bloemfontein Zoo, Henry Street, Bloemfontein Tel: +27 51 405 8483
Fax: +27 51 412 7011 E-Mail: daryl.barnes@mangaung.co.za Website: www.mangaung.co.za

AT THE HEART OF IT ALL

Figure 5.5.3: Permission to collect mosquitoes at the Bloemfontein Zoo.

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Henry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: henryG@daff.gov.za
Reference: 12/11/1/4

G I du P Terblanche
Department of Medical Microbiology and Virology
Faculty of Health Sciences, University of the Free State,
Francois Retief Building, Bloemfontein
Email: gduplerblanche@gmail.com

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984)

Dear G I du P Terblanche

Your email dated 30 May 2016 requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

I am pleased to inform you that permission is hereby granted to perform the following study, with the following conditions :

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. All potentially infectious material utilised or collected during the study is to be destroyed at the completion of the study. Records must be kept for five years for audit purposes. A dispensation application may be made to the Director Animal Health in the event that any of the above is to be stored or distributed;
3. No mosquito collection may be done in areas under veterinary restriction;
4. All mosquitoes must be transported dead;
5. The mosquito samples must be packaged and transported in accordance with International Air Transport Association (IATA) requirements and/or the National Road Traffic Act, 1996 (Act No. 93 of 1996);
6. All handling of mosquito samples must be done in a bio hazardous cabinet.

Title of research/study: Arboviruses in the Free State Province, South Africa
Researcher: G I du P Terblanche
Your Ref./ Project Number:
Institution: University of the Free State
Our ref Number: 12/11/1/4

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH

Date: 2016 -06- 01

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Harry Gelaw • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HarryG@daff.gov.za
Reference: 12/11/1/4

G I du P Tarblanche
Department of Medical Microbiology and Virology
Faculty of Health Sciences, University of the Free State,
Francois Retief Building, Bloemfontein
Email: gdupertblanche@gmail.com

RE: DISPENSATION ON SECTION 20 APPROVAL IN TERMS OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984) FOR: "ARBOVIRUSES IN THE FREE STATE PROVINCE, SOUTH AFRICA"

A dispensation is hereby granted on Point 2 of the Section 20 approval that was issued for the above mentioned study (attached):

i) RNA extracted from mosquito tissues may be stored in a designated -80°C freezer within the Department of Medical Microbiology and Virology, Faculty of Health Sciences, University of the Free State;

ii) DNA extracted from mosquito appendages may be stored in a designated -20°C freezer within the Department of Medical Microbiology and Virology, Faculty of Health Sciences, University of the Free State;

iii) Complete intact mosquito specimens may be dried, pinned and kept in an entomological collection for identification purposes;

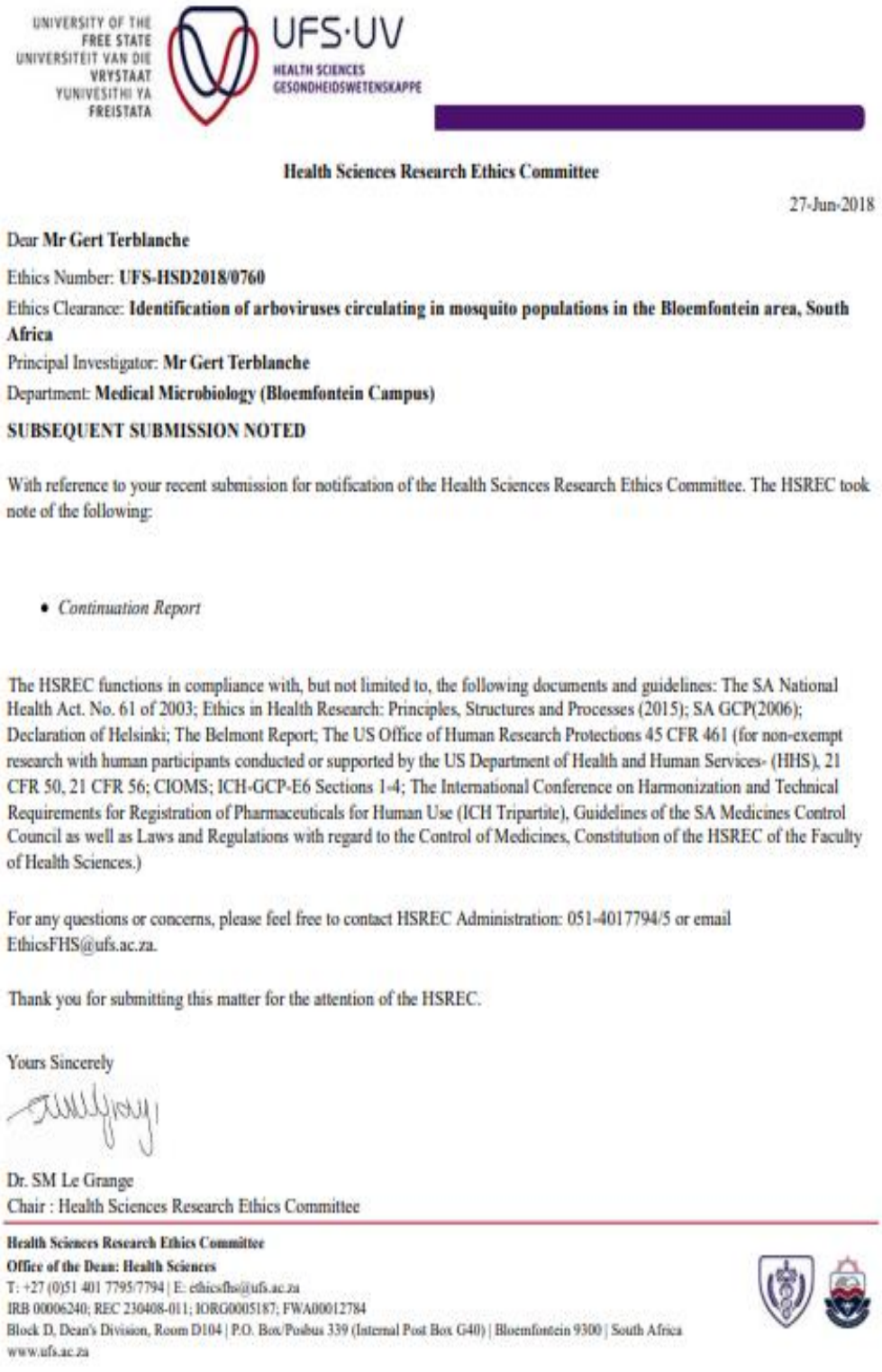
iv) Stored samples may not be outsourced or used for further research without prior written approval from DAFF

Kind regards,

DR. MPHO MAJA
DIRECTOR: ANIMAL HEALTH
Date: 2016-08-01

- 1 -

Figure 5.5.4: DAFF approval to perform the study.



UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

UFS·UV
HEALTH SCIENCES
GESONDHEIDSWETENSKAPPE

Health Sciences Research Ethics Committee

27-Jun-2018

Dear **Mr Gert Terblanche**

Ethics Number: **UFS-HSD2018/0760**

Ethics Clearance: **Identification of arboviruses circulating in mosquito populations in the Bloemfontein area, South Africa**

Principal Investigator: **Mr Gert Terblanche**

Department: **Medical Microbiology (Bloemfontein Campus)**

SUBSEQUENT SUBMISSION NOTED

With reference to your recent submission for notification of the Health Sciences Research Ethics Committee. The HSREC took note of the following:

- *Continuation Report*

The HSREC functions in compliance with, but not limited to, the following documents and guidelines: The SA National Health Act. No. 61 of 2003; Ethics in Health Research: Principles, Structures and Processes (2015); SA GCP(2006); Declaration of Helsinki; The Belmont Report; The US Office of Human Research Protections 45 CFR 461 (for non-exempt research with human participants conducted or supported by the US Department of Health and Human Services- (HHS), 21 CFR 50, 21 CFR 56; CIOMS; ICH-GCP-E6 Sections 1-4; The International Conference on Harmonization and Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH Tripartite), Guidelines of the SA Medicines Control Council as well as Laws and Regulations with regard to the Control of Medicines, Constitution of the HSREC of the Faculty of Health Sciences.)

For any questions or concerns, please feel free to contact HSREC Administration: 051-4017794/5 or email EthicsFHS@ufs.ac.za.

Thank you for submitting this matter for the attention of the HSREC.

Yours Sincerely

Dr. SM Le Grange
Chair : Health Sciences Research Ethics Committee

Health Sciences Research Ethics Committee
Office of the Dean: Health Sciences
T: +27 (0)51 401 7795/7794 | E: ethicsfhs@ufs.ac.za
IRB 0006240; REC 230408-011; IORG0005187; FWA00012784
Block D, Dean's Division, Room D104 | P.O. Box/Posbus 339 (Internal Post Box G40) | Bloemfontein 9300 | South Africa
www.ufs.ac.za

Figure 5.5.5: Most recent Ethics Committee Approval letter.

5.6 Correct mosquito transport protocol

All field caught mosquitoes need to be inactivated in the field and cannot be transported alive from the site of collection to the laboratory. All mosquito species were added to containers containing RNAlater®. This inactivates the mosquitoes and ensures safe handling and transport. The mosquitoes were placed in a sealable container, which was placed in a second sealable container. The mosquitoes were transported directly to the NHLS laboratories at the Health Science faculty of the University of the Free State, Bloemfontein campus. Mosquitoes were identified and placed in individual micro-centrifuge tubes containing RNAlater®. Each tube was clearly labelled with species name, collection site and collection date. These tubes were placed in a -20°C freezer until needed for downstream reactions.