Insecticide susceptibility analysis of *Anopheles gambiae* complex in Vlakbult, Mpumalanga 2012/13

By

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A dissertation submitted in partial fulfillment of the requirements for the degree Master of Public Health in the Faculty of Health Sciences University of Pretoria Pretoria

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[redacted]

Declaration

I declare that the dissertation titled "**Insecticide susceptibility analysis of *Anopheles gambiae* complex in Vlakbult, Mpumalanga 2012/13**" which I hereby submit for the degree Master of Public Health to the University of Pretoria is my own original work and where other people's work has been used, it has been properly acknowledged and referenced. Furthermore, neither this work, nor any part of it, has been submitted to any other tertiary institution for any certificate, degree or diploma.

Full names of Student: Ntsieni Rahab Ramalwa

Signature

Signed: Date: 31/October/2013.

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Firstly, I would like to thank God for affording me an opportunity to study my MPH and to conduct my MPH research with the University of Pretoria. Most importantly, for always renewing my strength when I felt like I could not take it anymore.

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Lastly, I would like to give thanks to the South African Field Epidemiology and Laboratory Training Program (SAFELTP) staff for the financial support they supported me with .I will always be grateful for all the skills I have learnt through SAFELTP.

May God bless you and every work of your hands.

Dedication

This dissertation is dedicated to my husband, Khathutshelo Mathews Sekhwama and my daughter Selaelo Cynthia Sekhwama, for all the time that I have taken away from home, working on this research study while they kept me going, giving me love, strength and support. This work is also dedicated to my mother, Dorah Maphaha (20/June/1960 – 02/December/2013) for being the strongest woman I have ever known, a true friend, a shoulder to cry and lean on, a woman who strongly believed that education is the best tool to conquer this world and lastly, a woman who believed in me more than I believed in myself. Thank you Khathu, Selaelo and Mama for letting me know that I can make it.

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List of Acronyms

DDT……Dichlorodiphenyl-Trichloroethane

ELISA……Enzyme Linked Immunosorbent Assay

IRS……..Indoor Residual Spraying

KZN……KwaZulu-Natal

NICD……..National Institute of Communicable Diseases

NHLS……National Health Laboratory Services

PCR……Polymerase Chain Reaction

SAJS……..South African Journal of Science

VCRL……Vector Control Reference Laboratory

WHO……World Health Organisation

Executive summary

South Africa is one of the 34 malaria endemic countries that are currently targeting malaria elimination. Although South Africa is also listed as malaria endemic, malaria in South Africa is limited to the North-eastern three provinces of KwaZulu-Natal (KZN), Mpumalanga and Limpopo.

Anopheles arabiensis is the major malaria transmitting vector and the causative agent in over 90% of the malaria infections in South Africa had been and remains *Plasmodium falciparum.*

Many species of malaria vectors are known to belong to a 'species complex' and therefore look identical morphologically. Such species need to be identified further through the use of modern molecular methods such as Polymerase Chain Reaction (PCR). One such group is the *An. gambiae* complex, which comprises of eight members: *An. gambiae, An. coluzzii* and *An. arabiensis* (major malaria vectors), *An. merus, An. melas* and *An .bwambae* (minor or localized vectors) and *An. quadriannulatus* and *An. amharicus* are not known to transmit malaria.

This study focused on a new vector breeding site identified in Mpumalanga (Vlakbult) in 2011.The aims of this study were to: Obtain data on malaria vector prevalence in Vlakbult, Mpumalanga province (November 2012-January 2013), determine insecticide susceptibility status of *An. arabiensis* from Vlakbult and to determine sporozoite infectivity rate in wild caught *An.arabiensis* mosquitoes collected in Vlakbult from November 2012 –January 2013.

The study found that the breeding site in Vlakbult had *An. arabiensis that were* found in this breeding site are susceptible to 4% DDT and 0.05% deltamethrin and that the wild caught *Anopheles arabiensis* found in this area (Vlakbult) were not infected with *P. falciparum* parasites.

For the Mpumalanga malaria control program, these findings mean that there is a need for continuing entomological and vector susceptibility surveillance in order to inform malaria control and elimination efforts. Mpumalanga malaria control also needs to review their vector control activities in Vlakbult to ensure that the presence of *An. arabiensis* is addressed

appropriately since the presence of the malaria transmitting vector in Vlakbult poses a potential risk for an outbreak of malaria.

PART ONE: RESEARCH PROTOCOL

1.1 INTRODUCTION

Malaria is a parasitic disease that is spread to humans by a mosquito bite from an infected female *Anopheles* mosquito[1]. The WHO, World Malaria Report 2011 recorded an estimated 216 million cases of malaria in the year of which 81% were in the African region. In addition, the report also recorded an estimated 655 000 deaths of which 91% were in the African region.[2] In South Africa mainly three provinces, Limpopo, KwaZulu–Natal (KZN) and Mpumalanga, are affected by malaria (Figure 1). Malaria in South Africa is categorized as seasonal and unstable because it does not occur throughout the year, but between September and May, and also has the potential for causing outbreaks, hence unstable.[3]

There are four main African malaria vectors and three of these, *An. arabiensis, An. coluzzi* and *An. gambiae* belong to the *An. gambiae* complex.[4,5] The other main vector, *An. funestus* is a member of the *An. funestus* group.[4,5] The *An. gambiae* complex consists of eight species: *An. gambiae s.s*, *An. arabiensis*, An *merus, An. melas, An. quadriannulatus* , *An. amharicus* and *An. bwambae*[4,5,6] *Anopheles arabiensis* is one of South Africa's main malaria vectors. The other main historical vector is *An. funestus*, but this vector has since been eliminated from South Africa, except for its re-invasion during the 1999/2000 malaria epidemic. [7]

This study will mainly focus on *An. arabiensis* and due to space constrain, this is the only species that will be discussed in more detail. *Anopheles arabiensis* has a wide distribution in Africa ranging from Madagascar to Senegal.[4] This species preferably breeds in fresh, temporary, sunlit, rain water pools [4,5] and is more tolerant of higher temperatures (arid regions).[4] *Anopheles arabiensis* has a more opportunistic feeding behavior and can be anthropophilic and zoophilic as well as endophilic and exophilic, making them incompletely vulnerable to house-spraying

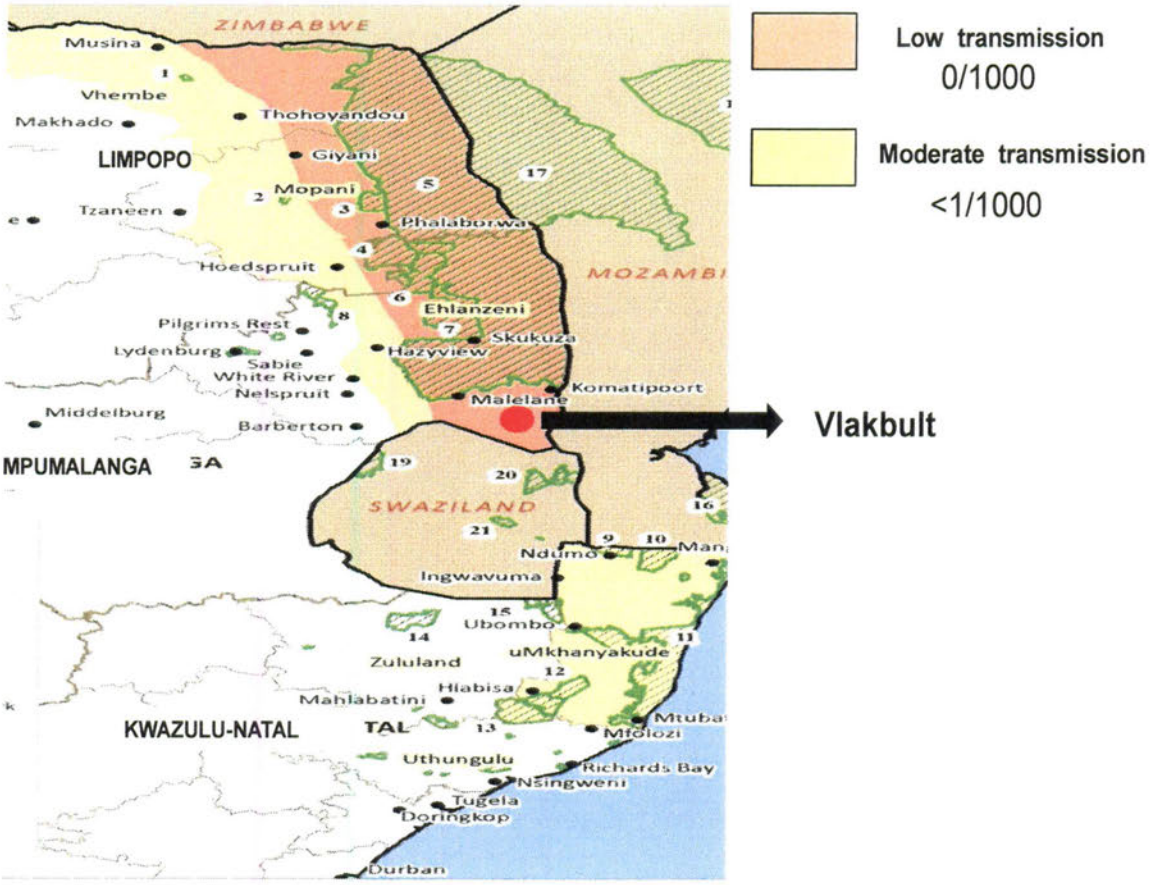

Figure 1: Malaria risk areas in South Africa, 2013, showing malaria low and moderate risk areas.

Malaria vector control in South Africa relies mainly on indoor residual spraying (IRS) with insecticides.[8] IRS in South Africa was introduced as early as 1945.[8] Dichlorodiphenyl-Trichloroethane (DDT) was introduced for malaria control in 1946 and used until 1996 when there was a change in policy regarding the use of DDT. [8] The pyrethroid, deltamethrin, replaced DDT; however, between 1996 and 2000, there was a dramatic increase in malaria cases despite continued efficient house spraying in KZN and Mpumalanga (Figure 2). DDT was reintroduced in March 2000.[8] South Africa is one of the countries that continues using DDT as an insecticide for vector control and the insecticide susceptibility status of vectors is a requirement by World Health Organizations (WHO).[9]

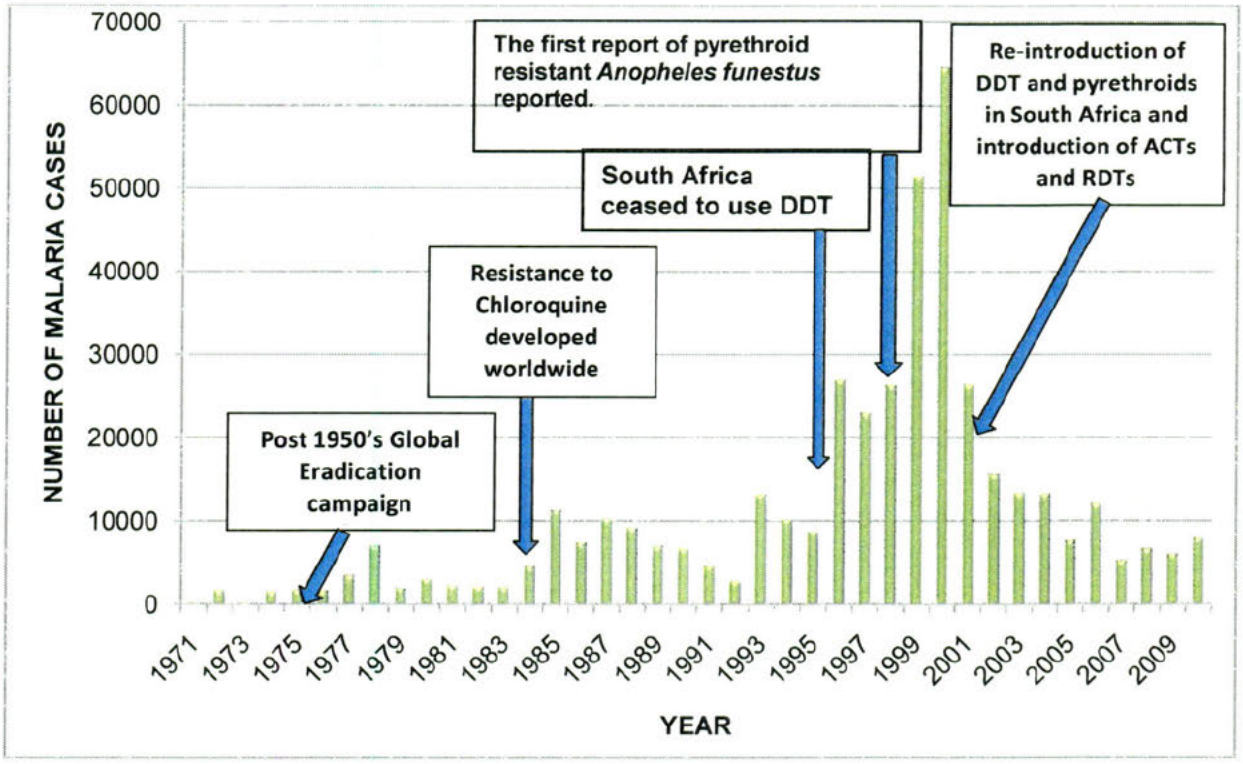

Figure 2: Malaria in South Africa, 1971-2010.[8]

There are four classes of insecticides that are available for public health purposes, namely: organochlorines, organophosphates, carbamates and pyrethroids.[10] However, insecticides resistance is a major concern in any malaria control program. During 1999/2000 South Africa experienced its worse malaria epidemic in over five decades when pyrethroids resistance in one of the main African malaria vectors, *An funestus*, was reported.[11] Insecticide resistance can develop rapidly and constant monitoring is imperative in a vector control program.[10]

Apart from pyrethroid resistance in *An. funestus*[11], resistance to DDT has also been detected in *An. arabiensis* in 2002.[12] Two years later in 2005 pyrethroid resistance in the same species was reported in KZN. [13]

Insecticide resistance is an inherited characteristic that allows an insect to survive a dose of an insecticide that would normally prove fatal and the insect therefore tolerates the doses of a toxicant that kills insects in a normal population of the same species[10]

Insecticide resistance is becoming more prevalent in many parts of the world due to reduction in the sensitivity of insect populations to the insecticides.[10] Constant monitoring and evaluation of insecticide resistance is needed. This study therefore aims to investigate insecticide susceptibility of the main vector, *An. arabiensis* in Vlakbult, Mpumalanga Province

Due to the fact that South Africa has one of the most effective malaria control programs on the continent, it is extremely difficult and time consuming to identify vector pockets. However, to monitor insecticide resistance, it is important to have access to wild vector populations. Recently, an entomologist from Mpumalanga malaria control program identified a vector breeding site at Vlakbult. [15] A relatively large number of *Anopheline* larvae were identified as *An. arabiensis*, this was therefore be the main locality of this study.

1.2 RESEARCH QUESTIONS

The study aimed to answer the following questions:

1. What species of *An.gambiae* complex were present at Vlakbult during 2012/2013?
2. Is the *An. gambiae* complex population at Vlakbult susceptible to insecticides that are currently used for vector control in Mpumalanga?

1.3 STUDY OBJECTIVES

3.1 To identify the species of *An. gambiae* complex collected at Vlakbult in 2012/2013.

3.2 To determine the susceptibility of detected mosquito species to insecticides (DDT and Pyrethroids).

3.3 To determine if *An. gambiae* complex from Vlakbult are malaria parasite infective.

1.4 METHODOLOGY

1.4.1 Study design

The study employed an observational study design, where all the species were collected and exposed to 4% DDT and Pyrethroids, using WHO insecticides impregnated papers for the experiment group and insecticide susceptible mosquitoes for controls.

1.4.2 Study setting

Mosquitoes that were used in this study were collected from Mpumalanga Province. Mpumalanga is administratively divided into three districts: Ehlanzeni, Nkangala and Gert Sibande.[14] For this study, field sampling was conducted from November 2012 to February 2013 at Enhlanzeni district in Vlakbult (Mpumalanga, South Africa) 25^0 38'43.3"S, 031^0 42' 00.2")).[15]

1.4.3 Sample collection

Samples were collected using four different methods: Larval collection, collections using carbon dioxide traps ($C0_2$ traps), pit traps collections and human landing catches.

1.4.3.1 Larval collections.

Larvae will be collected in Vlakbult, from a nearby river, using large scooping spoons, where water is stagnant. Larvae will be collected by dipping the scooping spoon into the water where mosquito larvae would have been identified and will be transferred into 5 litre buckets. Pasteur pipettes will be used to select larvae from water into larvae holding containers were larvae will be fed and transported to the Malaria Control Unit in Mpumalanga until the last day of collection where larvae will be transported to the Vector Control Reference Laboratory (VCRL) at the National Institute of Communicable Diseases (NICD).[16]

1.4.3.2 Collections from carbon dioxide-baited net traps

The mosquito trapping nets were planted on the ground near mosquito breeding sites and inside, the CO_2 in the form of dry ice was placed in the cool and allowed for emission. Mosquitoes that will get trapped on the nets will be collected using polystyrene cups and mouth aspirators.

1.4.3.3 Collections from human landing catches

Human landing catches were done by sitting on chairs on the open space, closer to the breeding sites. Each one involved in mosquito catching for the night will have a polystyrene cup and sucking tube to collect adult mosquitoes. The tops of the cups containing collected mosquitoes will be covered with gauze held in place by elastic rubber band to protect the mosquitoes from escaping. A small cotton wool soaked in 10% sugar solution will be placed on top of cups and gauze for feeding of captured mosquitoes. The cup containing mosquitoes will be kept cool in a cooler box with a freeze block.[16]

1.4.3.4 Mosquito collection from Pit Traps.

Searches for adult mosquitoes were conducted from manmade holes in a sand bank near mosquito breeding sites. The mosquitoes will be collected from the pits using sucking tubes and they will be transferred into polystyrene cups. The cup containing mosquitoes will be kept cool in a cooler box with a freeze block.[16]

All the samples (adult mosquitoes and larvae) collected will then be transported to the VCRL insectary at the National Institute for communicable diseases (NICD) in Johannesburg where mosquito rearing and laboratory tests will be performed. At the VCRL insectary, all wild collected mosquitoes will be placed in separate glass vials, containing a damp filter paper to facilitate oviposition and each mosquito will be given a unique identity number (continuing from VCRL mosquito lab numbers from routine tests). These parate egg lying tubes for oviposition are to keep each F_1 generation separate. Adults that emerge from the larval bowls will be cleared, given a lab number and be exposed to insecticides at age 3-5 days.[16]

1.4.4 Insecticide susceptibility tests

The test tubes with WHO insecticides impregnated papers will be prepared and placed on the susceptibility testing table overnight, together with control tubes (tubes with untreated papers). The following day, three days old adults (n=20-25) cleared from larval bowls will be exposed to 4% DDT and 0.05% deltamethrin (pyrethroid). Susceptibility tests will be conducted using WHO

insecticide-impregnated papers and test kits. A laboratory colony of *An. arabiensis (KGB)* which is known to be susceptible to all insecticides will be used as a control to ensure the reliability of impregnated papers.[17] In addition; negative controls will be used, wherein tubes consisting of mosquitoes will be exposed to untreated papers. Knockdown will be recorded for 1 hour. Firstly, recording will be done for every 5 minutes for the first 30 minutes followed by recording every 10 minutes thereafter for the remaining 30 minutes. After 1 hour, the mosquitoes were transferred from the exposure tube to a holding tube and the knockdown rate recorded. Mosquitoes were provided with a 10% sucrose solution and the final mortality was recording 24 hours post-exposure as defined by WHO criteria for determining susceptibility/resistance to diagnostic insecticide concentrations.[17]

1.4.5 Sporozoites/parasites detection

Only wild caught females will be analyzed for the presence of sporozoites. The heads and thoraces of wild female mosquitoes will be separated from the rest of the body and analyzed for the presence of malaria parasites (*P .falciparum*) using enzyme linked immunosorbent assay (ELISA) technique.[20] Negative controls consisting of unfed, colonized specimens of *An. arabiensis* will also be included, and synthetic peptide standardized against P. *falciparum* will be included on each plate as a positive control. Results will be analyzed using a microtitre plate reader at wavelength 405 nm.[18]

The vector mosquitoes collected from Vlakbult were assayed using the ELISA method to investigate their *P.falciparum* sporozoites rate and also, to give an indication of vector capacity.

1.4.6 Species Identification

Field-collected adults of *An. gambiae* complex mosquitoes were identified using morphological keys [4] and were further identified to species level using the polymerase chain reaction (PCR) assay of Scott et al. (1993). [19]

PCR reaction[19] consisted of the following reagents, 1.25µl 10x reaction buffer (100m M Tris-HCL pH 8.3, 1mM KCl), 1.25µl 10x dNTP, 0.5µl MgCl2 solution, 0.5µl Quad Primer, 1.0µl

each of UN, AG, AR, ME and QD primers, 4.9µldeonised H2O and 0.1µl Taq. A volume of 12.5 µl of the Master Mix was aliquoted into each 0.2 µl PCR and DNA added. Negative controls consisted of master mix. PCR tubes with contents were centrifuged for 20 seconds at 16K rpm to collect reaction mixture. Reaction was subjected to PCR cycling conditions of 95°C for 2 minutes initial denaturation, 30 cycles of 94°C for 30 seconds denaturation of DNA, 50°C for 30 seconds annealing of specific primers, 72°C for 30 seconds extension and a final auto extension of 72°C for 5 minutes.

After amplification, the samples were removed from PCR machine and stored in the freezer. 4µl of loading dye was added to the content of each tube and samples loaded in the well of the gel. Positive controls were loaded alphabetically (*An. arabiensis*, *An. gambiae*, *An. merus* and *An. quadriannulatus*) followed by negative controls to the end of the gel. Samples were loaded and gel electrophoresed at 100V/400mA for approximately 60 minutes. After electrophoresis, the gel was placed into the GeneSnap cabinet (Vacutec G-Box from Syngene) and photographs of the PCR products were taken.

Samples were scored by comparing the product size to those of the positive controls and molecular marker size. An *An. gambiae* PCR file was developed and saved on the computer where data analysis was conducted.

This study generated primary data which was entered and cleaned using personal laptop, data will be processed using excel spreadsheet, STATA for statistical analysis and Epi Info software. Data generated will be analyzed according to WHO criteria, which states that:

- 98-100% mosquito mortality 24 hours after exposure to insecticides indicates susceptibility,
- <98% suggests potential resistance and needs to be confirmed and
- < 90% mortality suggests resistance.[20]

1.5 DUMMY TABLE

Insecticide used: DDT

Family(siblings)	Family size	Insecticides used DDT	% Mortality
Family 1			
Family 2			
Family 3			
Family 4			
Family 5			
Family 6			
Family 7			
Family 8			

1.6 ETHICAL AND LEGAL CONSIDERATIONS

Malaria control program had given permission to proceed with the study in Mpumalanga after an entomologist from Mpumalanga malaria control program identified a vector breeding site at Vlakbult [19]. Permission to conduct this study was also obtained from the University of Pretoria Main ethics (attached in the apendices).

1.7 BUDGET

Item	Cost per item	Total cost
Field work by field workers	R115/worker/day	R115 X 3X 9=R3105
Lab work	PCR,ELISA, animal husbandry	R10 000
Travel	Covered through VCRU trips	R0 00
Accommodation	R600/night X 2 weeks	R12 000
GRAND TOTAL		R25 105

The funds for the study are available from the South African Field Epidemiology and Laboratory Training (SAFELTP) grant.

1.8 ACTIVITIES AND RESPONSIBILITIES

ITEM	Person responsible	Start date	End date
Entomology skills training	VCRL team	July 2012	December 2012
Mosquito collection	Mpumalanga Team, VCRL Team and Ntsieni	November 2012	January 2013
Rearing	Ntsieni (and the VCRU team)	November 2012	January 2013
Bio-assay test	Ntsieni(and the VCRL team)	November 2012	March 2013
Data entry and cleaning	Ntsieni	January 2013	March 2013
Data analysis	Ntsieni	February 2013	February 2013

1.9 GANTT CHART

Task	Dec 2012	January 2012	February 2013	March 2013	April 2013	May 2013	June 2013	July 2013	August 2013
Draft protocol									
APC submission									
Ethics submission									
protocol approval									
Mosquito collection									
Mosquito rearing									
Bio-assay test									
Data entry									
Data analysis									
Report Writing									
Dissertation submission to University of Pretoria									
Publication									

1.10 REFERENCES

1. Nchinda T.C. (1998) Malaria, a re-emerging disease in Africa. World Health Organization. *Emerg Inf Dis.* 4(3), 398-403.

2. World Health Organization.(2011). *World Malaria Report* Geneva, Switzerland. http//www.who.int/malaria/publications/world_malaria_report_,2011/en/. (Accessed 10 June 2012).

3. Maharaj R, Mthembu DJ and Sharp BL. (2005). Impact of DDT reintroduction on malaria transmission in KwaZulu-Natal. *S. Afr. Med. J.* **95**(11).

4. Gillies M.T. and Coetzee M. (1987). A supplement to the Anophelinae Africa South of the Sahara. *S. Afr. Inst. Med. Res.* Publication no.55.

5. Gillies M. and DeMeillon B. (1968). The Anophelinae of Africa South of the Sahara. *S. Afr. Inst. Med. Res.* Publication no.54.

6. Hunt, R.H., Coetzee, M. and Fettene, M. (1998). The *Anopheles gambiae* complex: a new species from Ethopia. *Trans. R. Soc. Trop. Med. Hyg.* 92(2), 231-235.

7. Hargreaves K., Koekemoer L.L., Hunt R.H., Brooke B.D., Mthembu J. And Coetzee M. (2000). *Anopheles funestus* resistant to pyrethroid insecticides in South Africa. *Med. Vet. Entomol.* **14**, 181–189.

8. National Department of Health: *Malaria policy Pretoria.* South Africa. 2011. www.doh.gov.za/docs/policy/2011/malaria_prevention.pdf. (Accessed 15 June 2012).

9. Ahrens R and Weber C. (2009). DDT and the Stockholm convention. States on the edge of non-complience. *Pan Germany.* http://www.pan.germany.org/download/ddt/PAN_G_DDT_Study_EN.pdF. (Accessed 10 February 2014)

10. World Health Organization. (2012). Global Plan for Insecticide Resistance Management in Malaria Vectors (GPIRM). Geneva:WHO.http://www.who.int/iris/handle/10665/44846. (accessed 3 November 2012).

11. Hagreaves K, Koekewmoer L.L, Hunt R.H, Brooke B.D, Mthembu J and Coetzee M. (2000). Anopheles funestus resistant to pyrethroid insecticides in South Africa. *Med. Vet Entomol.* 14,181-189.

12. Hargreaves K, Hunt R .H, Brooke B.D, Mthembu J, Weeto M.M, Awolola T.S and Coetzee M. (2003). *Anopheles arabiensis* and*An.quadriannulatus* resistance to DDT in South Africa. *Med. Vet Entomol.*17,417-422.

13. Mouatcho J.C., Munhenga G., Hargreaves K., Brooke B.D., Coetzee M., Koekemoer L.L. (2009). Pyrethroid resistance in major African malaria vector Anopheles arabiensis from Mamfene, northern Kwazulu-Natal, South Africa. *S. Afr. J.Sci.* **105, 127-131.**

14. Ngomane L. and de Jager C. (2012). Changes in malaria morbididty and mortality in Mpumalanga province, South Africa (2001-2009): a retrospective study. Malaria J*mal Jl.***11**:19.

15. Menzane Frans Mbokazi . (2012). A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in partial fulfillment of the requirements for the degree of MSc in Biology and Control of African Disease Vectors. Johannesburg.

16. Sri Lanka Anti Malaria campaign. (2009) Guidelines for entomological surveillance of malaria vectors in Sri Lanka. http://www.malariacampaign.gov.lk/downloads/revised%20%guidelines20%for%entomologica%20%surveillance. (Accessed 13 February 2014).

17. World Health Organization.(1998). *Test procedures for insecticide resistance monitoring in malaria vectors, Bio-efficacy and persistence of insecticides on treated surfaces.Geneva: WHO.* http//www.who.int/iris/handle/10665/64879 (Accessed 11 June 2012).

18. Wirtz R.A., Zavala F., Charoenvit Y., Campbell G.H., Burkot T.R., Schneider I., Esser K. M., Beaudoin R.L. and André R.G. (1987). Comparative testing of *Plasmodium falciparum* sporozoite monoclonal antibodies for ELISA development. *Bull. WHO.* **65**, 39–45.

19. Scott, JA, Brogdon,WG, Collins, FH.(1993). Identification of single species of the Anopheles gambiae complex by the polymerase chain reaction. *Am. J. Trop. Med. Hyg.* 49,520–529.

20. Koekemoer L.L, Spillings B.L, Christian R.N, Lo T.M, Kaiser M.L, Northon R.A.I et. Al,. (2011) Multiple Insecticide Resistance in *Anopheles gambiae* (Diptera:Culicidae) from Ponte Noire, Republic of Congo. *J. Vector. Borne. Dis.* 11(8),1193-200.

PART TWO: JOURNAL ARTICLE

2.1 cover letter

Faculty of Health Sciences
School of Health Systems and Public Health
HW Snyman Building (North)
31 Bophelo Road
Gezina
Pretoria
30 October 2013

The Editor
South African Journal of Science (SAJS)

REF: SUBMISSION OF MANUSCRIPT

Dear Sir/Madam,

Please find attached our manuscript entitled **"Insecticide susceptibility analysis of *Anopheles gambiae* complex in Vlakbult, Mpumalanga 2012/13"** by Ntsieni Rahab Ramalwa et., al a research article for consideration for publication in your journal.

We believe the results presented in the manuscript provide a guide to malaria control efforts in Mpumalanga in terms of vector control. Mpumalanga will know what interventions are needed for vector control in Vlakbult because there are malaria transmitting vectors detected in this area and also, locally acquired cases have been reported in Vlakbult after our study detected *An. arabiensis* in this area.

All authors listed have approved the manuscript and declared no competing interests. We declare that this manuscript has not been published in any scientific journal and is not being considered for publication by another journal.

Thank you for your consideration. Please address all correspondence to me by e-mail: ntsieni.sekhwama@gmail.com

Yours sincerely,

Ntsieni Rahab Ramalwa

2.2 Manuscript

Insecticide susceptibility analysis of *Anopheles gambiae* complex in Vlakbult, Mpumalanga 2012/13

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2.3 Abstract

Recent entomological surveillance studies revealed the presence of *Anopheles arabiensis,* a major malaria transmitting vector within the *Anopheles gambiae* complex in Vlakbult, an area in Mpumalanga Province. The aim of the study was to obtain baseline data on prevalence and insecticide susceptibility of vector mosquitoes present in Vlakbult. This experimental study was conducted based on the World Health Organization (WHO) bioassay testing procedure. Mosquitoes were collected and identified using morphological keys and Polymerase Chain Reaction. Mosquitoes morphologically identified as *Anopheles gambiae* complex were exposed to 4% DDT and 0.05% deltamethrin and mortality recorded after 24-hours. An enzyme-linked immunosorbent assay (ELISA) test was then conducted on these mosquitoes to detect sporozoites. A total of 130 mosquitoes were collected and identified. Results revealed that 56 (43%) were *Anopheles gambiae* complex, of which 35 (63%) were identified as *Anopheles arabiensis* and 4 (7%) were identified as *Anopheles merus.* There was 100% mortality within 24 hours of exposure to 4% DDT and 0.05% deltamethrin. The ELISA test revealed zero parasite prevalence (0/32). This study concludes that the presence of *An. arabiensis* in the mosquito population of Vlakbult poses a potential risk for an outbreak of malaria as these are known malaria transmitting vectors. The Mpumalanga malaria control program needs to increase their surveillance and review mosquito control strategies in the Vlakbult area.

Key words: *Anopheles gambiae* complex, insecticides, susceptibility

2.4 Introduction

Malaria remains one of the most serious tropical diseases in the world[1]. The 2012 World Malaria Report estimates 216 million cases of malaria worldwide[1] with 81% occurring in the African region. In addition, the report also recorded an estimated 655 000 deaths of which 91% were in the African region.[1] In South Africa three provinces, Limpopo, KwaZulu-Natal (KZN) and Mpumalanga, are affected by local malaria transmission.[2] Malaria in South Africa is categorized as seasonal, with peak transmission occuring between September and May, with the potential of outbreaks.[2]

South Africa is targeting malaria elimination by the year 2018, wherein the country aims for zero recorded locally acquired cases of malaria infections.[3-5]The country has made enormous progress in controlling malaria transmission over the past 100 years and the most important lesson learned has been the need for constant vigilance and surveillance of malaria vectors and parasites.[6]

The main essential step in a malaria vector control programme is the accurate identification of mosquitoes involved in malaria transmission so that the limited resources are not wasted on other mosquitoes that do not transmit malaria.[6] This starts with separating *Anopheles* mosquitoes from other culicine mosquitoes and then identifying species within the genus *Anopheles* so as to separate vectors from non-vectors.[7,8] Many species are known to belong to a 'species complex' and therefore look identical morphologically. Such species can be identified further through the use of modern molecular methods such as PCR.[9,10] One such group is the *An. gambiae* complex, which comprises of eight members: *An. gambiae, An. coluzzii* and *An. arabiensis* (major malaria vectors), *An. merus, An. melas* and *An .bwambae* (minor or localized vectors) and *An. quadriannulatus* and *An. amharicus* are not known to transmit malaria.[11,12]

Although South Africa has one of the most effective malaria control programs on the continent, it relies on the use of insecticides for vector control.[6] South Africa is one of the countries that continue to use DDT in combination with pyrethroids for indoor residual spraying (IRS).[6] In order to ensure that the insecticides chosen for malaria vector control remain effective, routine WHO insecticide susceptibility assays should be carried out on the local vector populations.[13]

The aims of this study were to : obtain data on malaria vector prevalence in Vlakbult, Mpumalanga province (November 2012-January 2013), determine insecticide susceptibility status of *An. arabiensis* present in Vlakbult and to determine sporozoite infectivity rate in wild caught *An.arabiensis* mosquitoes collected in Vlakbult from November 2012 –January 2013.

Fig.1. A map of Mpumalanga, showing the five municipalities (Mbombela, Bushbuckridge, Thaba Chweu, Umjindi and Nkomazi) of Ehlanzeni district.[15] The Vlakbult region is marked by the red star.

2.5 Materials and Methods

2.5.1 Study Site

Researchers from the NICD and Mpumalanga malaria control program including field workers, field epidemiologist and entomologists, chose the Vlakbult area 25^0 38' 43.3" S, 31^0 42' 00.2" E (Fig 1), as the main site for this study.

Entomology surveillance conducted in Vlakbult in 2011 detected a vector breeding site from this area and identified *An. arabiensis*, a major malaria transmitting vector (Table 1).[16] Comparing to other mosquito species collected in all new breeding sites, Vlakbult produced *An. arabiensis* in abundance. [14]

Table 1: Composition of species collected from new breeding sites in Mpumalanga, October – December 2011.[14]

New Breeding sites	*An. arabiensis*	*An. merus*	*An. quadriannulatus*	*An. rivulorum*	TOTAL
Komati Town	0	3	1	0	4
Mangweni	0	6	0	0	6
Boschfontein	0	2	0	0	2
Magudu	0	1	0	0	1
Mgobodi	2	2	1	0	5
Mzinti	2	8	0	0	10
Tamahawk	3	5	1	7	16
Vlakbult	23	2	0	0	25
TOTAL	30	29	3	7	**69**

Vlakbult is a village situated in Nkomazi municipality that falls under Enhlanzeni district of Mpumalanga province in South Africa (Fig: 1).[15] During the period of the study, 640 cases were reported in Nkomazi municipality, 10 of which were reported from Vlakbult (Mpumalanga malaria control, unpublished data)

2.5.2 Data collection

We collected samples from the Vlakbult one full week per month during November 2012, December 2012 and January 2013. Adult mosquitoes were collected using three different methods: collections from carbon dioxide ($C0_2$) baited traps, pit trap collections and from human landing catches (HLC). Larval collections were also conducted from stagnant water a near flowing river in Vlakbult.

Adult *An. funestus* group and *An. gambiae* complex specimens were collected and transferred into polystyrene cups. To protect the mosquitoes from escaping, the cups were covered with gauze held in place by elastic rubber bands. Mosquitoes had access to 10% sucrose solution. All the collected specimens (adult mosquitoes and larvae) were transported to the Vector Control Reference Laboratory (VCRL) insectary at the National Institute for communicable diseases (NICD) in Johannesburg.

The wild female mosquitoes were placed in separate glass oviposition vials. The females had access to damp filter paper for oviposition and 10% sucrose. Eggs were reared as iso-female lines and emerged adults (F_1 generation) were combined into cages. Adults had access to 10% sugar water and were analyzed for insecticide susceptibility when they were between 3 - 5 days old.

2.5.3 Insecticide susceptibility tests

The exposure tubes containing insecticide impregnated papers, sourced from WHO, were prepared and placed on the susceptibility testing table overnight, together with control tubes. The following day, mosquitoes aged between 3 and 5 days old were exposed to 4% DDT and 0.05% deltamethrin.[16] Controls included a laboratory colony of *An. arabiensis* (KGB) which is known to be susceptible to all insecticides was used as a control to ensure the reliability of impregnated papers and mosquitoes that were exposed to untreated papers (no insecticide).[16]

Initial insecticide knockdown was recorded after a one hour exposure period, following which the 24 hour post exposure mortality was calculated.[16] The WHO criteria for determining susceptibility/resistance to diagnostic insecticide concentrations was used, which states that a

98–100% mortality rate 24 hours post insecticide exposure is indicative of susceptibility, < 98% requires further investigation, and < 90% confirms vector resistance to insecticides.[16]

2.5.4 Species identification

The adult mosquitoes captured at the site, and adults resulting from the larval collections, were identified to species group or complex using morphological keys. [7,8,9,17,18,21] Species level identifications of the *An. gambiae* complex specimens was carried out by Polymerase Chain Reaction (PCR) according to Scott *et al.*[9] PCR using the rDNA method developed by Koekemoer *et al.*, was used to identify members of the *An. funestus* group.[19] DNA extractions for the aforementioned PCR protocols were conducted using a salt precipitation method.[9]

2.5.5 Plasmodium falciparum Sporozoites Detection

Wild female mosquitoes captured at the site were analyzed for the presence of sporozoites. The heads and thoraces were separated from the rest of the body and analyzed for the presence of *P. falciparum* circumsporozoite protein, using an indirect ELISA technique.[20] Negative controls consisting of unfed, colonized specimens of *An. arabiensis* were also included, and synthetic peptides standardized against *P. falciparum* were included on each plate as a positive control. Results were analyzed and after the first ELISA test, specimens that tested positive were retested by boiling the specimen and re-conducting the ELISA technique to confirm infection of mosquitoes with sporozoites.[20]

2.6 Results

2.6.1 Mosquito collections and identification

A total of 130 mosquitoes were captured during the study period. Identification of collected mosquitoes by morphological keys identified 62 mosquitoes belonging to the *An. gambiae* complex [47.6%], 26 [20%] belonging to the *An. funestus* group, 3 [2.3%] as other Anophelines and 39 [30%] as Culicinae. PCR revealed that 39 [62.9%] of the *An. gambiae* complex collected

were *An. arabiensis,* 8 [12.9%] *An. quadriannulatus*, 4 [7.1%] *An. merus* and 11 [8.5%] could not be identified at the species level.

The *An. funestus* group mainly consisted out of *An. vaneedeni* (61.5%; 16/26) followed by *An. rivolurum* (6/26; 23.1%) and *An. leesoni* (4/26; 15.4%). The specimens identified as *An. marshalli, An. demeilloni* contributed to less than 5% of the total sample collected (Table 2).

Table 2: Mosquitoes captured at the Vlakbult, Mpumalanga region of South Africa during three time periods d from November 2012 to January 2013.

Complex	Species	Number collected	Percentage (%)
Anopheles gambiae Complex	Unidentified (to species level)	11/130	8.5%
	An. arabiensis	39/130	30%
	An. quadriannulatus	8/130	6.2%
	An. merus	4/130	3.1%
Anopheles funestus Group	*An. vaneedeni*	16/130	12.3%
	An. leesoni	4/130	3.1%
	An. rivulorum	6/130	5.4%
Other Anophelines	*An. demeilloni*	2/130	2.3 %
	An. marshalli	1/130	
Culex group	Culex sp.	39/130	30%
TOTAL		130 mosquitoes	100%

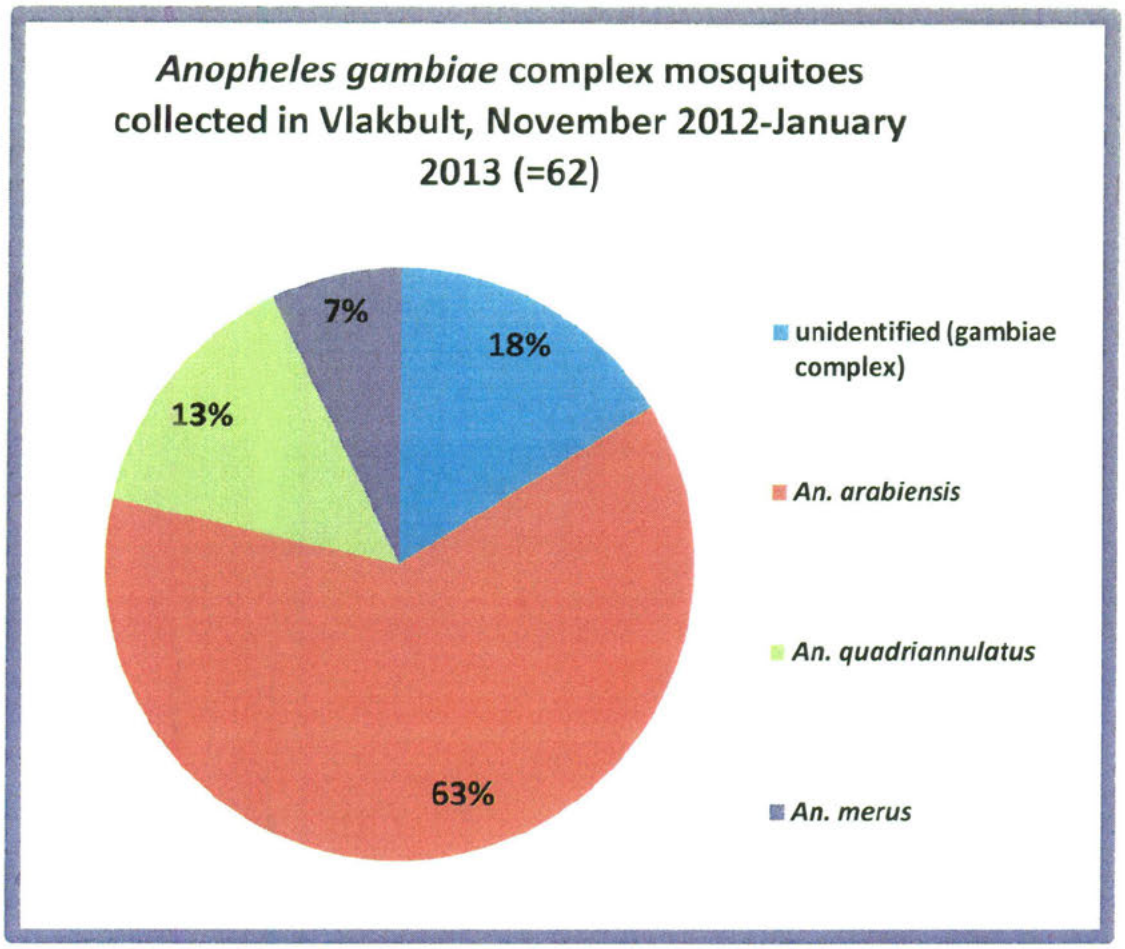

Fig.2. Mosquito species from *An. gambiae* complex, showing majority of mosquitoes to be *An.arabiensis*. 16% were could not be identified by PCR but were morphologically identified as mosquitoes from *An. gambiae* complex.

Table3. Mosquito collections by method of collection, November 2012-January 2013: Vlakbult, Mpumalanga Province of South Africa.

	METHO -D				
SPECIES	**Human landing catches**	**Larvae**	**Carbon dioxide trap**	**Pit traps**	**Total**
An arabiensis	2	32	5	0	**39**
An. quadriannulatus	2	4	2	0	**8**
An. merus	1	2	1	0	**4**
unidentified (An. *gambiae* complex)	5	5	1	0	**11**
An. vaneedeni	7	1	7	1	**16**
An. leesoni	1	0	2	1	**4**
An. rivulorum	5	0	0	1	**6**
An. demeilloni	1	0	1	0	**2**
An. marshalli	0	0	1	0	**1**
***Culex* species(non vector for malaria)**	34	4	1	0	**39**
Total	**58**	**48**	**21**	**3**	**130**

The collection method that yielded the most *An. arabiensis* specimens was the larval collection from stagnant water near a flowing river at the Vlakbult site. Collection from pit traps yielded three mosquitoes and the $C0_2$ baited trap method yielded double the number of mosquitoes collected from HLC method.

2.6.2 Susceptibility Tests

The captured mosquitoes (larvae that were reared through to adults) were exposed to only two classes of insecticides, Organochlorides (4% DDT) and pyrethroids (0.05% deltamethrin) because these are the two insecticides that are used in Mpumalanga for IRS and because of the small sample size collected. In total there were 28 captured *An. arabiensis* mosquitoes derived from the larval collections, and 50 controls exposed. The post 24 hour recordings showed 100% mortality, indicated on figure 2 below).

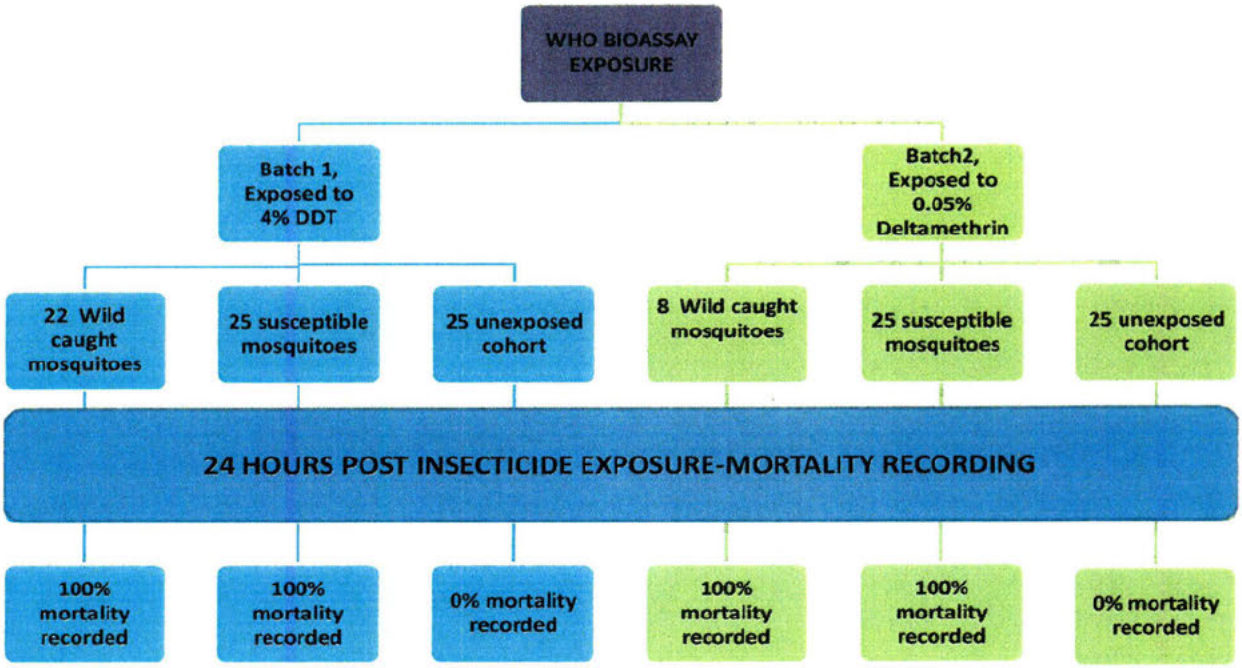

Figure 2: Results obtained from WHO standard susceptibility assays.

2.6.3 Parasites detection

A total of 32 captured adult *An. arabiensis* mosquitoes were tested for the presence of *P. falciparum* circumsporozoite protein using the ELISA technique. 32/32 (100%) mosquitoes tested negative for *P. falciparum*.

2.7 Discussion

Mpumalanga Province is one of the malaria endemic provinces of South Africa[2]. A vector breeding site was identified in 2011 at Vlakbult, an area which is considered malaria low risk within Mpumalanga and entomological surveillance was conducted during 2011.[14]

The results from this study revealed the presence of *An. arabiensis* mosquitoes. *Anopheles arabiensis* are highly efficient vectors and are responsible for malaria transmission in large parts of sub–Saharan Africa.[7,8,18] *Anopheles arabiensis* possesses both the anthropophilic and zoophilic feeding patterns and rests both indoors and outdoors. [7,8] This exophilic and endophilic behavior presents a great challenge to vector control programmes that only use IRS, because it means that other feasible and acceptable strategies will have to be developed for outdoor control.[7,17,21]

The majority (28/35 [80%]) of *An. arabiensis* mosquitoes that were collected for this study were collected using larvae collection method. This could mean that for the Mpumalanga malaria control, larviciding may be an additional malaria control intervention to current IRS interventions in Vlakbult. In addition, *An. arabiensis* outdoor-resting behavior[7,8] further emphasizes the potential suitability of larviciding for vector control within this area.

Three methods were used for collection of adult mosquitoes in this study, namely: collections from human landing catches, $C0_2$ baited traps and collections from pit traps. When comparing these methods, collection from human landing catches yielded more than double the number of mosquitoes collected using $C0_2$ baited traps collection method and three mosquitoes were collected using the pit trap collection method from this new breeding site.

Anopheles arabiensis showed full susceptibility to 4% DDT and 0.05% deltamethrin based on the WHO recommendation. Although the observed mortality percentage was 100% on both the DDT and deltamethrin, future monitoring for insecticide resistance is critical.

WHO recommends a sample size of 100 mosquitoes (per species, excluding controls) to be exposed per insecticide for conclusive results.[16] However, this study only exposed 22 *An. arabiensis* to 4% DDT and 8 *An. arabiensis* to 0.05% deltamethrin, And for each exposure, 50 controls were included. The small sample size highlights that entomological surveillance needed for extensive periods of time to ensure sufficient data are collected to inform the malaria control efforts in Mpumalanga.

A study done by M.F.Mbokazi 2011[14], also showed very low numbers of mosquitoes (*An. arabiensis*, *An. merus* and *An. quadriannulatus)* when collecting from this site (Vlakbult) in October, November and December 2011.[14] We therefore suspect that this new vector-breeding site has low mosquito populations. However, within the low numbers of mosquitoes collected, the abundant vector is *An. arabiensis*.[14]

Vector incrimination, through the detection of circumsporozoite proteins, revealed that none of the mosquitoes collected for this study were infected with *P. falciparum*. This finding should however be interpreted with caution. The sample size for this study was small and hence if a lower entomological infection rate is present in this population, it might not have been detected due to the limited sample size

Although the parasite infectivity rate was zero percent, the mosquito population in this area is made up of both major (*An. arabiensis*) and minor (*An. merus*) malaria vector mosquitoes and thus the risk of malaria transmission will continue to be present in this area. In March 2013, three locally transmitted malaria cases were reported from Vlakbult (Mpumalanga Malaria Control, unpublished data).

2.8 Conclusion

This study highlights that *An. arabiensis* is present in the Vlakbult area in Mpumalanga, and suggests that *An. arabiensis* is the dominant malaria vector. This vector is variable in its resting and feeding behaviors reducing the efficacy of an IRS only control strategy. [7,18,22] This study concludes that the major malaria transmitting vector is present in Vlakbult and the *An.arabiensis* present are susceptible to 4% DDT and 0.05% deltamethrin. None of the mosquitoes subjected to ELISA technique were infected with *Plasmodium falciparum* parasites. The presence of a major malaria transmitting vector in Vlakbult poses a potential risk for an outbreak of malaria. South Africa strives towards malaria elimination by 2018, and Mpumalanga province strives to maintain its malaria case fatality rate below 0.5% and its incidence of malaria to < 100 per 100 000 people per year[23]. To achieve these goals, malaria vector control and surveillance needs to be intensified, including the continuation of routine insecticide susceptibility studies.

2.9 Acknowledgements

We would like to acknowledge the following institutions and individuals for their contribution in this study: South African Field Epidemiology and Laboratory Training Programme, Vector Control Reference Laboratory of the National Institute of communicable diseases, University of Pretoria, National Department of Health and Mpumalanga Malaria Control team.

2.10 Disclosure statement

The authors declare no conflict of interest

Author contributions

N.R. Ramalwa conducted the work and wrote the first and subsequent drafts of the manuscript. B.L. Spillings and SV Oliver provide laboratory support and comments on the manuscript. E. Misiani provided comments on the manuscript. D.P. Moonasar, C. de Jager and A. Mabuza provided administrative support and comments on the manuscript. L.L. Koekemoer- Provided laboratory support, data analysis and comments on the first and subsequent copies of the manuscript. South African Field Epidemiology and Laboratory Training Program (SAFELTP) provided financial support for the project through the National Health Laboratory Service (NHLS)

2.11 References

1. World Health Organization. (2012). *World Malaria Report* .World Health Organization, Geneva:WHO,Switzerland. http//www.who.int/malaria/publications/world_malaria_report_,2012/en. (accessed 15 July 2013).

2. Maharaj R., Mthembu D.J. and Sharp B.L. (2005). Impact of DDT re-introduction on malaria transmission in KwaZulu-Natal. *S. Afr. Med. J.* **95**,871-874.

3. Cotter C, Sturrock H.J and Hsiang M.S. (2013) The changing epidemiology of malaria elimination: New strategies for new challenges. *Lancet* (in press). [http:dx.doi.org/10.1016/s0140-6736(13)60310-4].

4. World Health Organization. Malaria Elimination. (2007). *A field manual for low and ModerateEndemiccountries*.Geneva:WHO, Switzerland. http:/whqlibdoc.who.int/publications/2007/9789241596084_eng.pdf. (accessed 26 November 2013).

5. South African National Department of Health (2012). Malaria elimination strategy for South Africa 2012-2018. Pretoria: NDoH, 2012.

6. Blumberg L., Moonasar D., Coetzee M., Kruger P., Hunt R., Durrheim D et.al,. (2013) .Malaria in South Africa. Moving from control to elimination. *S. Afr. Med. J.* **103**(10):767-806.

7. Gillies M.T. and Coetzee M. (1987). A supplement to the Anophelinae Africa South of the Sahara. *S. Afr. Inst. Med. Res. Publication no.55.*

8. Gillies M. and DeMeillon B. (1968). The Anophelinae of Africa South of the Sahara. . *S. Afr. Inst. Med. Res. Publication no.54.*

9. Scott JA, Brogdon WG, Collins FH. (1993). Identification of single specimens of the anopheles gambiae complex by the polymerase chain reaction. *Am JTrop Med and Hyg.* **49**,520-529.

10. Koekemoer L.L., Kamau L., Hunt R.H. and Coetzee M. (2002). A cocktail polymerase chain reaction assay to identify members of the Anopheles funestus (Diptera: Culicidae) group. *Am J Trop Med Hyg.* **66**,804-811.

11. Hunt RH, Coetzee M and Fettene M. (1998). The *Anopheles gambiae* complex: A new species from Ethiopia. *Trans .R .Soc. Trop. Med .Hyg.* **92** (2):231-235.

12. Coetzee M., Hunt R.H., Wilkerson R.,Torre A.D., Coulibaly M.B., and Besansky N.J.(2013). *Anopheles coluzzii* and *Anopheles amharicus*, new members of the *Anopheles gambiae* complex. Zootaxa **3619**(3):246-274.

13. World Health Organization. (2012).Global Plan for Insecticide Resistance Management in Malaria Vectors (GPIRM). Geneva: WHO, 2012. http://www.who.int/iris/handle/10665/44846. (accessed 26 November 2013)

14. Menzane Frans Mbokazi (2012).A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in partial fulfillment of the requirements for the degree of MSc in Biology and Control of African Disease Vectors. Johannesburg .

15. Enhlanzeni District Municipality GIS unit. (2008). Also available at: http://www.enhlanzeni.co.za/index.php?option

16. WHO (2013).*Test procedures for insecticide resistance monitoring in malaria vectors.* World Health Organization, Geneva.

17. Ntomwa, B.N., Usuku, P., Govere, J.N., Manga, L., Koekemoer, L.L., Hunt, R.H. and Coetzee, M. (2006). Distribution of the *Anopheles gambiae* Giles *s.l.* complex in Namibia and susceptibility to insecticides used for malaria control. *Afr. Entomol.* **14**,404-406.

18. Coetzee M. And Hunt R.H. (1998). Malaria at its southern-most fringe in Africa. *Research and Reviews in Parasitology*. **58**,175-179.

19. Koekemoer L.L., Kamau L., Hunt R.H. and Coetzee M. (2002). A cocktail polymerase chain reaction assay to identify members of the Anopheles funestus (Diptera: Culicidae) group. *Am J Trop Med Hyg.* **66**,804-811.

20. Wirtz R.A., Zavala F., Charoenvit Y., Campbell G.H., Burkot T.R., Schneider I., Esser K. M., Beaudoin R.L. and André R.G. (1987). Comparative testing of *Plasmodium falciparum* sporozoite monoclonal antibodies for ELISA development. *Bull. WHO.* **65**, 39–45.

21. Masendu, H.T., Hunt, R.H., Koekemoer, L.L. and Brooke, B.D. (2005). Spatial and temporal distributions and insecticide susceptibility of malaria vectors in Zimbabwe. *Afr. Entomol.* **13**,25-34.

22. Sinka M.E., Bangs M.J., Menguin S., Coetzee M., Mbongo C.M., Hemmingway J., et.al, (2010). The dominant anopheles vectors of human malaria in Africa, Europe and Middle East: Occurance data, distribution maps and bionomic précis. *Parasites and Vectors.* **3**:117.

23. Ngomane L. and de Jager C. (2012). Changes in malaria morbidity and mortality in Mpumalanga Province, South Africa (2001-2009): A retrospective study. *Malar J.* **11**:19.

APPENDICES

Appendix 1: University of Pretoria Ethics Approval

Appendix 2: Mpumalanga department of health Ethics approval

Appendix 3: SAJS Manuscript Guidelines

APPENDICES

APPENDIX 1

University of Pretoria Ethics approval letter

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 20 Oct 2016.
- IRB 0000 2235 IORG0001762 Approved dd 13/04/2011 and Expires 13/04/2014.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

30/10/2013

Approval Certificate
New Application

Ethics Reference No.: 126/2013

Title: Insecticides susceptibility analysis on *Anopheles gambiae* complex in Vlakbult, Mpumalanga (November 2012-March 2013)

Dear Rahab Ntsieni Ramalwa

The **New Application** for your research received on the 1/03/2013, was approved by the Faculty of Health Sciences Research Ethics Committee on the 30/10/2013

Please note the following about your ethics approval:
- Ethics Approval is valid for 1 year.
- Please remember to use your protocol number (126/2013) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:
Standard Conditions:
- The ethics approval is conditional on the receipt of 6 monthly written Progress Reports, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

pp

Professor Werdie (CW) Van Staden
MBChB MMed(Psych) MD FCPsych FTCL UPLM
Chairperson: Faculty of Health Sciences Research Ethics Committee

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

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APPENDIX 2

Mpumalanga Department of Health Ethics approval letter

MPUMALANGA PROVINCIAL GOVERNMENT

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Department of Health

Litiko Letemphilo | Umnyango WezaMaphilo | Departement van Gesondheid

Enquiries: Themba Mulungo (013) 766 3511

18 September 2013

Ms. Ntsieni Ramalwa
P.O.Box 17538
Lyttelton
Centurion
0140
South Africa

Dear Ms. Ntsieni Ramalwa

APPLICATION FOR RESEARCH & ETHICS APPROVAL: INSECTICIDES SUSCEPTIBILITY ANALYSIS OF ANOPHELES GAMBIAE COMPLEX IN VLAKBULT, MPUMALANGA FROM NOVEMBER 2012-MARCH 2013

The Provincial Research and Ethics Committee has approved your research proposal in the latest format that you sent.

Kindly ensure that you provide us with the soft and hard copies of the report once your research project has been completed.

Kind regards

Kekeka

Mr. Molefe Machaba
Research and Epidemiology

15/09/2013
Date

APPENDIX 3

South African Journal of Science Manuscript guidelines to authors

ASSAf
SOUTH AFRICAN
Journal of Science

Guidelines for Authors

The *South African Journal of Science* accepts articles from any source on the understanding that they are the original work of the authors named and that they are being offered only to the *South African Journal of Science*.

Various kinds and categories of article are welcome. (Please consult a recent issue of the journal for examples.) Research communications are of three kinds: Research Letters, Research Articles and Review Articles. Research Letters are shorter reports (normally no longer than 2000 words) and should be up-to-date accounts of interesting and noteworthy scientific developments. Although these reports may be concerned with very particular advances, they should be of wider than specialist interest. Research Letters are given priority in terms of rapid publication after acceptance. Research Articles are longer papers (normally no more than 6000 words in length). Here the criteria of intelligibility and wider interest are strictly applied. Review Articles (up to 6000 words long) should be up-to-date surveys of important current developments in science. Preference is given to concise, reader-friendly submissions.

Submission of manuscripts for consideration: Manuscripts should be submitted online at http://mc.manuscriptcentral.com/sajs Please ensure that you have complied with the guidelines and completed the publishing agreement (available from the login page) before you submit. Submissions that are incomplete or do not comply with the instructions will be returned.

Pre-submission enquiries: If you wish to enquire whether your submission might be suitable for consideration by the *South African Journal of Science*, please email the Editor-in-Chief at j.butleradam@gmail.com. All pre-submission enquiries must include a summary and a cover letter outlining the article's interest to a broad scientific readership.

Readability: As the journal has a multidisciplinary focus, authors are requested to write their manuscripts in a manner and style that is intelligible to specialists and non-specialists alike. Articles are judged by reviewers at the discretion of the editors. Contributions should therefore be written clearly and simply so that they are accessible to readers in other disciplines and to readers for whom English is not a first language.

Note: Please use UK spelling and not US spelling. If in doubt, consult the Oxford English Dictionary.

Format of compulsory cover letter: The cover letter should indicate briefly the significance of the work being reported. State the full name, title, affiliation and contact details (postal address, email, telephone and cell number) of each author. Please identify the author to whom all correspondence should be addressed.

Include a paragraph briefly summarising the nature of the contribution made by each of the authors listed, along the lines of the following:

> *Author contributions*: J.K. was the project leader, L.M.N. and A.B. were responsible for experimental and project design. L.M.N. performed most of the experiments. P.R. made conceptual contributions and S.T., U.V. and C.D. performed some of the experiments. S.M. and V.C. prepared the samples and calculations were performed by C.S., J.K. and U.V. wrote the manuscript.

Authors will be required during submission to provide the names and full contact details (including email) of three potential reviewers to evaluate the work (reviewers should not be people with whom the researcher has recently collaborated or published).

Title, summary and keywords page: The article's full title should contain a maximum of 95 characters (including spaces). Five keywords should be provided. Articles and letters should begin with a fully referenced summary paragraph of up to 250 words, aimed at readers in other disciplines. This paragraph should start with 2–3 sentences that provide an introduction to the field and the particular problem being investigated. This should be followed by a one-sentence statement of the authors' main findings (or

conclusions, in the case of a review paper); and a further 2–3 sentences placing these findings/conclusions in a general context so that readers are made aware of what the implications of these findings are.

Ethical guidelines: Submissions involving research conducted on human or nonhuman vertebrates should meet the highest standards regarding both the ethical consideration given and reporting of the procedures followed. Full details are necessary so that a non-specialist reader can appreciate the need for the research undertaken.

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- Journal titles should be abbreviated.
- If the journal has continuous page numbering, the month/issue number can be omitted.
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Author's surname Initials, Author's surname Initials. Title of article. Abbreviated journal title [serial on the Internet]. Year of publication month day [cited year month day];volume(issue):[number of pages]. Available from: URL

Article in press

Author's surname Initials, Author's surname Initials. Title of article. Abbreviated journal title. In press Year.

Article not in English

Author's surname Initials, Author's surname Initials. Title of article in original language [translated title in English]. Abbreviated journal title. Year of publication;volume(issue number):page numbers. Original language.

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Author/editor's surname Initials. Title of book. ed. [if not 1st]. City of publication: publisher's name; year of publication.

Chapter in a book

Author's surname Initials. Title of chapter. In: Editor's surname Initials, editor. Title of book. ed. [if not 1st]. City of publication: publisher's name; year of publication. p. xx–xx. [page numbers of chapter, separated by an en dash and not elided]

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Author's surname Initials. Title of article. Title of newspaper. Year month day;page/section.

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Author/Editor/Organisation's name. Title of the page [homepage on the Internet]. City of publication: publisher's name; year created [updated year month day; cited year month day]. Available from: URL

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