Anopheles gambiae Complex: Molecular Forms and Occurrence of The KDR Gene in Rural Southwestern Nigeria

NOUTCHA, M.A.E.[1,2] and ANUMUDU, C.I[1]
[1]Cellular Parasitology Programme (CPP), Department of Zoology,
University of Ibadan, Oyo State, Nigeria.
[2]Malaria Research and Training Centre (MRTC), Faculty of Medicine,
Pharmacy and Odonto-Stomatology (FMPOS),
University of Mali, Bamako, Mali.

Nig. J. Ent. 28: 7-16 (2011)

ABSTRACT. An investigation focusing on the molecular forms of *Anopheles gambiae* complex and occurrence of the knockdown resistance (kdr gene) was carried out from June to July 2001 and August to September 2002 at Igbo-Ora, Nigeria using aspirator and DNA analyses. Of the 127 *An. gambiae s. l.* analysed, there were 66 (51.9%) M and 61 (48.03%) S forms, both recorded in *An. gambiae s.s.* and *Anopheles arabiensis*. The forms were virtually sympatric throughout the study. Two of the *An. gambiae s.s.* examined harboured the kdr gene (RR, RS). The occurrence of the kdr gene at Igbo-Ora indicated the existence of the gene in the savanna woodland populations; earlier records were from the rain forest and Guinea savanna. The implications of these results, within the context of incipient speciation in *Anopheles gambiae* s.s. are discussed.

Keywords: *Anopheles gambiae,* molecular forms, kdr gene, Guinea savanna, southwestern Nigeria

INTRODUCTION

Anopheles gambiae complex and *Anopheles funestus* group represent the most efficient vectorial system available in the world for *Plasmodium falciparum* and account for over 95% of all infective bites in the West African sub-region (Coluzzi, 1984). The selective adaptation to changes in the environment, the availability and characteristics of local host populations can drive a speciation process in vectors; this may explain the appearance of reproductively isolated sibling species that manifest unique biological characteristics (Klowden and Zwiebel, 2005). The Tropical Diseases Research (TDR) programme on Molecular Entomology advocates a thorough understanding of mosquito population genetics as an essential tool for the eradication of malaria (WHO, 1999). *An. gambiae* s.l. is a complex of sibling taxa characterized by various paracentric inversions, three of which are sympatric in West Africa. Coluzzi *et al.* (1979) identified five chromosomal forms of *An. gambiae*, designated, by a non-Linnean

Corresponding author;
Aline Noutcha, PhD.
Contact Address: Department of Animal and Environmental Biology, University of Port Harcourt, P.M.B. 5323 Choba, Port Harcourt, Rivers State, Nigeria.
Email: naemekeu@yahoo.com

nomenclature, as Bamako, Bissau, Forest, Mopti and Savanna. Three of these, namely Bamako, Mopti, and Savanna exist and occur in sympatry with *An. arabiensis* at many locations in Mali. The efficiencies of these chromosomal forms as malaria vectors reflect their specific adaptations to eco-ethological parameters of their environment (Coluzzi *et al.*, 1979). Favia *et al.* (1997) classified these members into M and S molecular forms of *An. gambiae* s.s. The kdr gene which contributes to resistance to pyrethroids in *An. gambiae* s.s. is widespread in West Africa (Gentile *et al.*, 2004; Della Torre *et al.*, 2005; Santolamazza *et al.*, 2008; Awolola *et al.*, 2007).

Unfortunately, a comprehensive map of the distribution of human malaria vectors in the West-African sub-region is very limited (WHO, 2005). This necessitated a concerted effort at the comprehensive vectorial mapping in countries of the sub-region. Parasitological and immunological investigations, as a component of a comprehensive epidemiological study on malaria at Igbo-Ora, a rural community in south western Nigeria, were initiated in 1991 (Nwagwu *et al.*, 1998). Igbo-Ora was the site to be used for malaria vaccine trials. It was against the background of dearth of data on the malaria vectors that entomological studies commenced in 2001; results on entomological indices (man biting, anthropophilic and entomologic Inoculation rates) and sibling species composition of *Anopheles gambiae* s. l. had been reported (Noutcha and Anumudu, 2009, 2010). This paper is on molecular forms and occurrence of the kdr gene in *An. gambiae* s. l., the dominant anopheline.

MATERIALS AND METHODS

Study area

Igbo-Ora is located 7.4333N and 3.2833E, at the boundary of the savanna woodland and rainforest (Fig 1), with an average annual temperature of 26.14°C and an annual rainfall of 1317 mm (Encarta, 2005). It is approximately 70km west of the university town of Ibadan. Contrary to the description by Lawrence (1965), the town is strewn with streams that serve as breeding grounds for mosquitoes. The town consists of 170 hamlets, grouped into seven villages (Ajegunle, Igbole, Idofin, Igbo-ora, Iberekedo, Packo and Sagaun), with 60,000 inhabitants, dominated by Yorubas. The major activities are subsistence farming, small-scale cocoa and tobacco farming, and petty trading among middle class citizens. Additional information on the area has been documented (Noutcha and Anumudu, 2009; 2010).

Field Studies

Collections were made daily for a week, in each of four months: June, July 2001 and August, September 2002, during the malaria transmission seasons. These were undertaken from 85 houses in 2001 and 72 in 2002 in four villages (Ajegunle, Igbole, Igbo-ora, Sagaun). All the anophelines found resting on surfaces in selected rooms were visually identified based on their resting position (Gordon and Lavoipière, 1978) and, systematically aspirated with a WHO-designed simple aspirator, modified from the mouth aspirator model of Coluzzi and Petrarca (1973). All collections were made in the morning, 08.00-11.00hrs, in rooms where people had slept the previous night, before they vacated the rooms. Upon capture, the mosquitoes were stored in paper cups, capped with nylon netting; one cup was used per room or house, depending on the mosquito density. Before handling at the field, mosquitoes were put on ice for about 10 minutes to incapacitate them and transferred into a Petri-dish for proper morphological identification with a hand lens as members of the *An. gambiae* complex. The length of the

palps, which are as long as the proboscis was used to confirm members of the genus *Anopheles* (Gordon and Lavoipière, 1978). Smooth 3-banded palps, black legs with white yellow speckles, pale brown, hairy abdomen (Gillet, 1972; Gillies and Coetzee, 1987) and patterns on wings (Holstein, 1960; Diagne *et al.*, 1994; Robert, 2003) were used to separate *An. gambiae* s. l. from other species. The mosquitoes caught in a house were then transferred into a labeled 50 ml-Corning tube and kept in the shade for 3-6 hours to ensure complete digestion of their blood meal. They were classified for gonotrophic state: unfed, fed, half-gravid and gravid, based on abdominal appearance (Detinova, 1963), fixed in Carnoy's fixative (100% ethanol: glacial acetic acid, 3:1) and freeze- stored at -20 to -70°C. Prior to mosquito processing at the lab, they were re-sorted with a pair of flexible tip forceps in a Petri-dish under a binocular dissecting microscope (Bunton Instruments Co. Incorporation, Rockville, MD. USA), and later dissected into various parts (head-thorax, blood meal, abdominal carcass, ovaries) based on their gonoptrophic stage. They were subsequently stored at -70 to -20°C in 0.2 ml Eppendorf tubes with Carnoy's fixative for PCR analyses.

Laboratory Analyses: Laboratory analyses were undertaken at the Malaria Research and Training Centre, Bamako, Mali, July, 2003-April, 2004.

DNA Extraction for PCR Analyses: Mosquito DNA was extracted from abdominal carcasses and from head-thorax crushes used for CSP- ELISA by the method of Collins *et al.* (1987). The DNA was used for species identification of the *An. gambiae* complex (Scott *et al.*, 1993), the molecular forms (Favia *et al.*, 2001) and the determination of the kdr gene (Martinez-Torres *et al.*, 1998).

Polymerase Chain Reaction Tests: The primers for *An. gambiae* molecular forms (Favia *et al.*, 2001), and the kdr gene (Martinez-Torres *et al.*, 1998) were purchased from Invitrogen; DNA amplifications were done with PTC-100HB and PTC-200 DNA Engines (MJ research, Inc. Watertown, MA.USA).

Mosquito DNA extracted was amplified, using the following conditions for *Anopheles gambiae* s.l. Molecular forms (M & S) identification a modified protocol from Favia *et al.* (2001) was used. The optimal amplification conditions were 94°C, 10min (Enzyme Activation); 94°C (Denaturation); 63°C (Annealing); 72°C (Extension); 30s each for 25cycles and 94°C, 7min for the identification of the M and S molecular forms of *An. gambiae* s.s. with four sets of primers (forward, reverse, Mop int, B/S int) yield 727bp (M) and 475bp (S) products respectively (Favia *et al.*, 2001).

The PCR assay for kdr resistance used the four primers AgD1-AgD4 (Martinez-Torres *et al.*, 1998). AgD1 and AgD2 flank the kdr mutation and amplify a 293bp product as an internal control. AgD3 binds only to the resistant kdr allele and pairs with AgD1 to amplify a 195bp fragment. AgD4 binds only to the susceptible allele and pairs with AgD2 to amplify a 137bp fragment (Norris, 2002).

Data Analyses: Data were analyzed using the Chi Square Test (χ^2- test).

RESULTS

A total of 1789 mosquitoes, 809 in 2001 and 980 in 2002 were caught from 91 houses (45 in 2001 and 46 in 2002). Morphological identification yielded 989 anophelines: 490 in 2001 and 499 in 2002. PCR analyses of the identified anophelines recorded 802 (81.09%) *An. gambiae* s. l. (420 in 2001 and 382 in 2002) (Table 1).

Table 1: **Numbers of PCR- Identified sibling species of *Anopheles gambiae* s.l.* at Igbo-Ora**

		Anopheles gambiae s. l.*		
Study Period		*Anopheles gambiae* s. s.	*Anopheles arabiensis*	Total
2001	June	100 (90.00%)	11 (10.00%)	111
	July	276 (89.32%)	33 (10.68%)	309
2002	August	197 (95.63%)	9 (4.37%)	206
	September	159 (90.34%)	17 (9.66%)	176

* 187 morphologically identified specimens could not be further characterized.

Table 2: **Monthly Frequency of Molecular Forms at Igbo-Ora in 2001 and 2002**

	Study Period					
Molecular	2001			2002		
Forms	June	July	Total	August	September	Total
M	1(1.85%)	53(98.15%)	54(100%)	5(41.67%)	7(58.33%)	12(100%)
S	11(30.55%)	25(69.44%)	36(100%)	15(60%)	10 (40%)	25(100%)
Total	**12 (13%)**	**78 (87%)**	**90 (100%)**	**20 (54%)**	**17(46%)**	**37(100%)**

The 1108 randomly- selected mosquitoes tested for molecular forms by a modified PCR technique yielded results in 127. Among the

Table 3: **Frequency of Molecular Forms within the *Anopheles gambiae* s.l. at Igbo-Ora**

	An. gambiae s.l.			
Molecular Forms	*An. arabiensis*	*An. gambiae* s.s.	Non- identified	Total
M	6 (9.1%)	45 (68.2%)	15 (22.7%)	66 (6.12%)
S	5 (8.2%)	44 (72.1%)	12 (19.7%)	61 (5.66%)
	8.66	70.07		

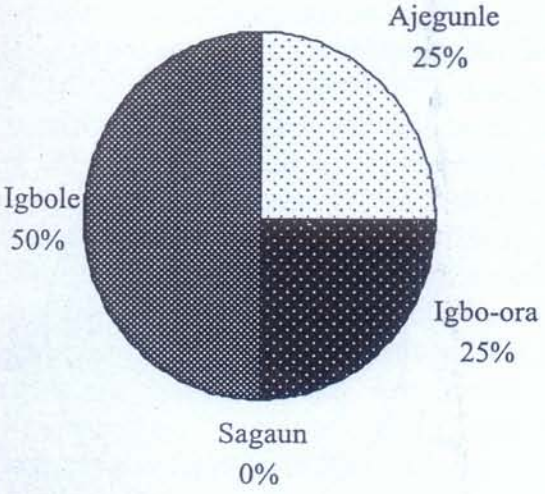
Non-identified refers to the 27 (21.3%) M or S forms not attributable to either species.

127, there were 66 (51.97%) M and 61 (48.03%) S forms, when pooled over the 2-year period (Table 2). The variation in the proportions of the M and S forms over this 2-year period was not significant ($\chi^2 = 0.24$, $df=1$, $p= 0.62$). The difference in the relative proportions of the M and S forms among villages over the 2-year period was significant ($\chi^2 = 0.3$, $df=1$, $p=0.85$). In 2001, higher numbers of the M form were identified, in contrast to 2002 when more S form were recorded. In 2001, both M and S forms were most abundant at Igbole and Igbo- ora, and

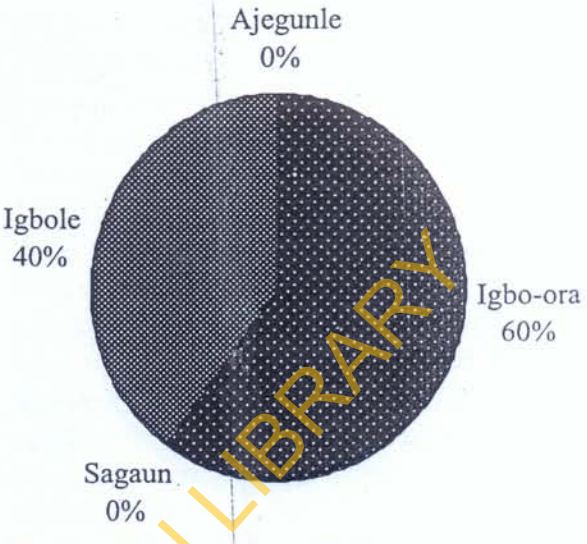
Table 4: **Distribution of the kds/kdr genes in *Anopheles gambiae* s.l. at Igbo-Ora**

Study Period			*An. gambiae* s.l.			
Year	Months	Alleles	*An. gambiae* s.s.	*An. arabiensis*	Non identified	Total
2001	June	SS	100 (*78.1%*)	11 (*8.6%*)	17(13.3%)	128 (27.0%)
	July	SS	276 (*79.8%*)	33 (*9.5%*)	37 (10.7%)	346 (73.0%)
	Subtotal		376 (51.5%)*	44 (62.9%)*	54 (37%)*	474 (50%)*
2002	August	SS	197 (*92.5%*)	9 (*4.2%*)	7 (3.3%)	213 (45.1%)
	September	SS	157 (*60.6%*)	17 (*6.6%*)	85 (32.8%)	259 (54.9%)
	Subtotal		354 (48.5%)*	26 (37.1%)*	92 (63%)*	472 (49.8%)*
	September	RS	**1(100%)****	**0**	**0**	**1(0.1%)****
		RR	**1(100%)****	**0**	**0**	**1(0.1%)****
Total			732 (77.2%)	70 (7.4%)	146 (15.4%)	948 (88.1%)
Not readable			91 (69.8%)	0	38 (21.7%)	129 (11.9%)
Grand Total			**843 (78.3%)**	**70 (6.5%)**	**164 (15.2%)**	**1077 (100%)**

Percents in italics are *An. gambiae* s.l. with susceptible gene (kds) based on monthly totals
*: *An. gambiae* s.l. with susceptible gene (kds) based on annual totals
**: *An. gambiae* s.s. with resistant genes (kdr) in September 2002

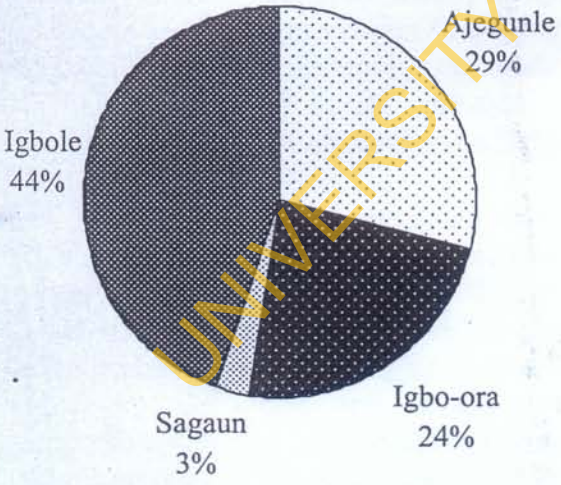


The M molecular form in *An. gambiae* s.s.

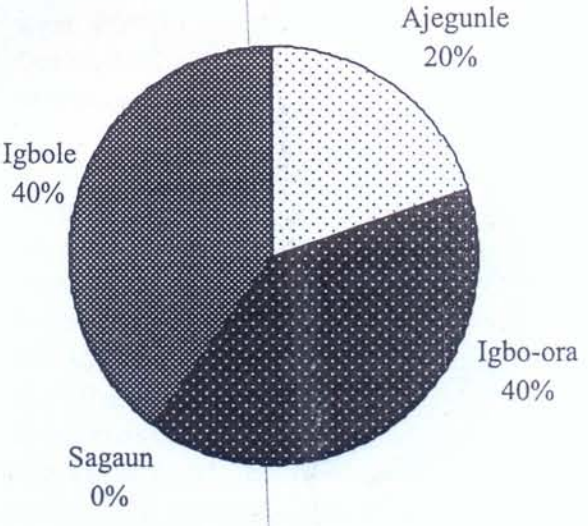


The M molecular form in *An. arabiensis*

a: The M Molecular Form, 2001



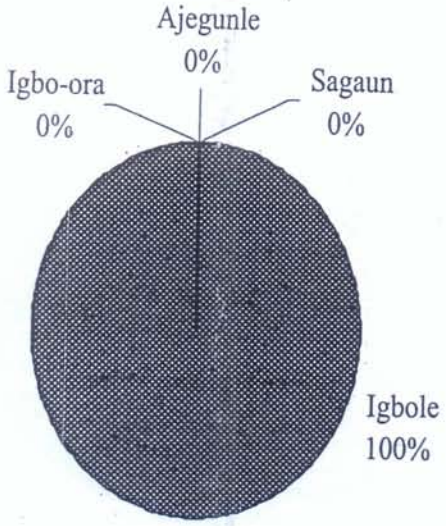
The M molecular form in *An. gambiae* s.s.



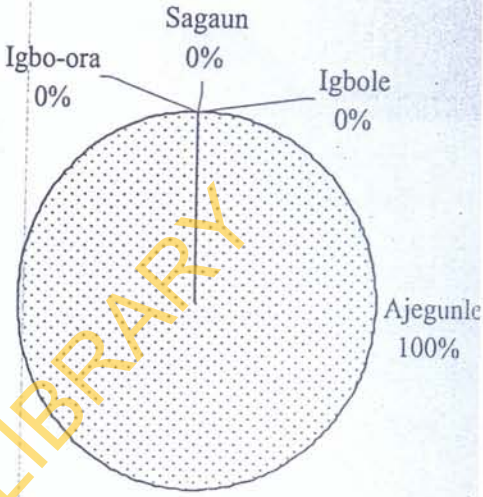
The M molecular form in *An. arabiensis*

b: The S Molecular Form, 2001

Figure 1: **Spatial distribution of the Molecular forms in Villages at Igbo-Ora**

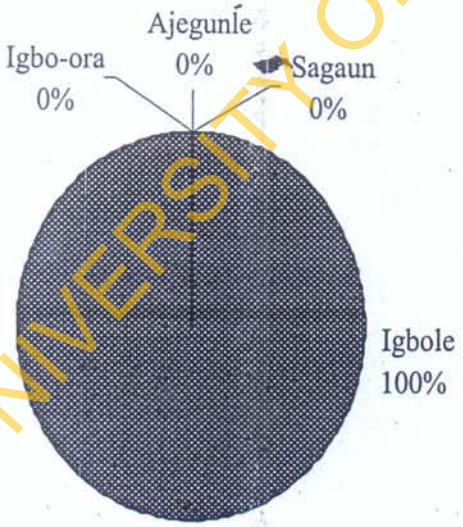


The M molecular form in *An. gambiae* s.s.

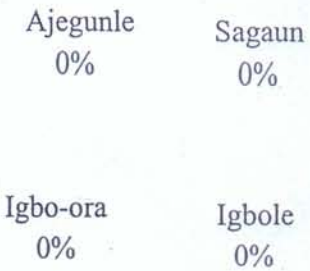


The M molecular form in *An. arabiensis*

a: The M Molecular Form, 2002



The M molecular form in *An. gambiae* s.s.



The M molecular form in *An. arabiensis*

b: The S Molecular Form, 2002

Figure 2: **Spatial distribution of the Molecular forms in Villages at Igbo-Ora**

least abundant at Ajegunle and Sagaun (Fig 1); but, in 2002, both forms were most abundant at Ajegunle (Fig 2). Among the 1077 mosquitoes tested for the knockdown resistance (kdr) gene, 946 (88%) had the susceptible (kds) gene (474 in 2001 and 472 in 2002). Some molecular forms (9.1% M and 8.2%S) were recorded among PCR identified *Anopheles arabiensis* (Table 3). Two specimens of *An. gambiae* s. s. caught in September 2002 from Igbo-ora harbored the kdr gene: one heterozygote resistant (RS) and one homozygote resistant (RR), (Table 4).

DISCUSSION

Possible reasons for the poor performance of the M and S assay are not available at this time. The non-significant difference in abundance between the M and S molecular forms in *An. gambiae* s. s. is similar to results obtained by Awolola *et al.* (2005) at Poka in the rain forest near Lagos. The spatial distribution of the two forms across the villages of Igbo-Ora was virtually similar. They were wholly sympatric at Igbole and Igbo-ora villages in 2001 and at Ajegunle in 2002. Literature is dominated by incipient speciation in *An. gambiae* s. s. (Fontenille *et al.*, 2005; Lehmann and Diabate, 2008).

The presence of the knockdown resistance (kdr) gene at Igbo-Ora complements the observations of Awolola *et al.* (2002; 2003) and Santolamazza *et al.* (2008), who recorded pyrethroid-resistant *An. gambiae s. s.* in the M form in the forest and savanna zones respectively. It was not possible to ascertain whether these heterozygous (RS) and homozygous (RR) resistant alleles were in the M or S form because the DNA amplification yielded no bands. Recent studies have indicated that the kdr gene is now widespread in the West African subregion (della Torre *et al.*, 2001; Gentile *et al.*, 2004; Santolamazza *et al.*, 2008). The basis for this type of resistance has been discussed by Martinez-Torrez *et al.* (1998). Although many studies in West Africa: Ivory Coast and Burkina-Faso (Martinez-Torrez *et al.*, 1998; Chandre *et al.*, 1999), Mali (Fanello *et al.*, 2002), Nigeria (Awolola *et al.*, 2005) had recorded the kdr gene exclusively in the S form; Weill *et al.* (2000) found the kdr gene in the M form in the Republic of Benin, at a location approximately 100km southwest of Igbo-Ora. They concluded that the kdr mutation in the M taxon was not an independent mutation. Although it was not possible to determine whether the resistant alleles were from the M or S form, recent studies (Reimer *et al.*, 2005) have shown that they are disproportionately present in M vs S forms. It was therefore likely that the 2 alleles were in the M form, particularly when Weill *et al.* (2000) recorded same from contiguous Benin Republic. Onyabe and Conn (2001) also suggested at least partial barriers to gene flow between the two forms in the Ivory Coast and other West African countries to the north and west. Further studies are in progress at Igbo-Ora on the distribution of the kdr gene between the two forms.

ACKNOWLEDGEMENTS

This project was mainly sponsored by a Third World Organization for Women in Science (TWOWS) Fellowship to **Aline NOUTCHA.** It was also supported by Training Funds from the Malaria Research and Training Center at the Faculty of Medicine, Pharmacy and Odontostomalogy (MRTC-FMPOS), University of Bamako, Mali and, the MIM/TDR project ID 980050 to the Cellular Parasitology Programme (CPP), Department of Zoology, University of Ibadan, Nigeria. Dr Douglas Norris of the John Hopkins Malaria Research Institute (JHMRI), Baltimore, USA, graciously contributed some reagents, Taq polymerase and DNA marker XIV, in 2003. Aline Noutcha is indebted to the MRTC

technical staff (Adama Sacko, Moussa Diallo, Boubacar Coulibaly and Dr Guimogo Dolo) for her training in mosquito laboratory and field techniques in 2001. The words of encouragement from Professors Yeya T. Touré, Mark Nwagwu and AMJ Oduola throughout Aline Noutcha's PhD training were inspirational. The cooperation and assistance provided by the chiefs and the entire Igbo-Ora community during the field work are gratefully acknowledged.

REFERENCES

Awolola T. S., Brooke B. D., Hunt R. H. and Coetzee M. (2002). Resistance of the malaria vector *Anopheles gambiae* s.s. to pyrethroid insecticides in Southwestern Nigeria. *Annals of Tropical Medicine and Parasitology* 96 (8), 849-852.

Awolola T. S., Brooke B. D., Koekemoer L. L. and Coetzee M. (2003). Absence of the kdr mutation in the molecular M form suggest different pyrethroid resistance mechanisms in the malaria vector mosquito *An. gambiae s.s. Tropical Medicine and International Health* 8 (5), 420-422.

Awolola T. S., Oduola A. O., Oyewole I. O., Obansa J. B., Amajoh C.N., Koekemoer L. L., and Coetzee M. (2007). Dynamics of knockdown pyrethroid insecticide resistance alleles in a field population of *Anopheles gambiae* s. s. in south-western Nigeria. *Journal of Vector Borne Diseases* 44 (3), 181-188.

Awolola T. S., Oyewole I., Amajoh C. N., Idowu E. T., Ajayi M.B., Oduola A., Manafa O. U., Ibrahim K. Koekemoer L. L. and Coetzee M. (2005). Distribution of the molecular forms of *Anopheles gambiae* and knockdown resistance gene in Nigeria. *Acta Tropica* 95(3), 204-209.

Chandre F., Mangin S., Brengues C., Dossou-Yovo J., Darriet F., Diabaté A., Carnevale P. and Guillet P. (1999). Current distribution of a pyrethroid resistance gene (kdr) in Anopheles gambiae complex from West Africa and further evidence for reproductive isolation of the Mopti form. *Parassitologia* 41, 319-322.

Collins F. H., Mendez M. A., Rasmussen M. O., Mehaffey P. C., Besansky N. J. and Finnerty. (1987). A ribosomal RNA gene probe differentiates member species of the *Anopheles gambiae* complex *American Journal of Tropical Medicine and Hygiene* 37, 37-41.

Coluzzi M. (1984). Heterogeneities of the vectorial malaria system in tropical Africa and their significance in malaria epidemiology and control. *Bulletin World Health Organization* Supplement 62, 483-97.

Coluzzi M. (2002). *Plasmodium falciparum* en Afrique subsaharienne: spéciation récente des vecteurs, évolution de la pathogenèse/ contrôle de la maladie, et capacité vectorielle *In:* Susceptibilité aux Maladies Infectieuses, Edition Scientifiques et Médicales Elsevier.

Coluzzi M. and Petrarca V. (1973). Aspirator with paper cup for collecting mosquitoes and other insects. *Mosquito News* 33, 249-250.

Coluzzi M., Sabatini A., Petrarca V. and Di Deco M. A. (1979). Chromosomal differentiation and adaptation to human environments in the *An. gambiae* complex. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 73, 483 - 497.

Della Torre A., Fanello C., Akogbeto M., Dossou-yovo J., Favia G., Petrarca V. and Coluzzi M. (2001). Molecular evidence of incipient speciation within *An. gambiae* s. s. in West Africa. *Insect Molecular Biology* 10(1), 9-18.

Della Torre A., Tu Z., and Petrarca V. (2005). On the distribution and genetic

differentiation of *Anopheles gambiae* s.s. molecular forms. *Insect Biochemistry and Molecular Biology* 35(7), 755-769.

Detinova T. S. (1963). Méthodes à appliquer pour classer par groupe d'âge les diptères présentant une importance médicale. *OMS Série Monographe* 47, 226.

Diagne N., Fontenille D., Konate L., Faye O., Lamizana M. T., Legros F., Molez J. F. and Trape J. F. (1994). Les anophèles du Sénégal : Liste commentée et illustrée. *Bulletin de la Société de Pathologie Exotique* 87, 267-77.

Encarta, (2005). The World Atlas. www.encarta.com 9 Fanello C., Santolamazza F. and Della Torre A. (2002). Simultaneous identification of species and molecular forms of the *An. gambiae* complex by PCR-RFLP. *Medical and Veterinary Entomology* 16, 461-464.

Favia G., Della Torre A., Bagayoko M., Lanfrancotti A., Sagnon N., Touré Y. T. and Coluzzi M. (1997). Molecular identification of sympatric chromosomal forms of *Anopheles gambiae* and further evidence of their reproductive isolation. *Insect Molecular Biology* 6, 377-383.

Favia G., Lanfrancotti A., Spanos L., Sidén-Kiamos I. and Louis C. (2001). Molecular characterization of ribosomal DNA (rDNA) polymorphisms discriminating among chromosomal forms of *An. gambiae* s. s. *Insect Molecular Biology* 10, 9-23.

Fontenille D., Cohuet A., Awono-Ambene P., Kengne P., Antonio-Nkondjio C., Wandji C., Simard F (2005). Malaria vectors from the field to genetics. Research in Africa. *Revue Epidémiologique de Santé Publique* 53 (3):283-290.

Gentile G., Santolamazza F., Fanello C., Petrarca V., Caccone A. and della Torre A. (2004). Variation in an intron sequence of the voltage-gated sodium channel gene correlates with genetic differentiation between *Anopheles gambiae* s. s. molecular forms. *Insect Molecular Biology* 13, 371-377.

Gillet J. D. (1972). Common African Mosquitoes. Heinemann. London 106pp.

Gillies M. T. and Coetzee M. (1987). A Supplement to the Anophelinae of Africa South of the Sahara. The South African Institute of Medical Research. Johannesburg.

Gordon B. M. and Lavoipiere M. M. J (1978). Entomology for students of medicine. Blackwell Scientific Publishers, Oxford. 352pp.

Holstein M. (1960). "Guide pratique de l'anophélisme en Afrique Occidentale Française" *Direction Générale de la Santé Publique, Service d'Hygiène Mobile et de Prophylaxie. 55 pp. Office de la Recherche Scientifique d'Outre- Mer (ORSTOM). Dakar.*

Klowden M. J. and Zwibel L. J. (2005). Vector Olfaction and behaviour *In* Biology of Disease Vectors. W. C. Marquardt (editor) Elsevier Academic Press. 2nd Edition Pp 277-287.

Lawrence B. R. (1965). Medical Entomology at Igbo-Ora, Western Nigeria. *Journal of Nigerian Medical Association* 2, 198-205.

Lehmann T., Diabate A. (2008). The molecular forms of *Anopheles gambiae*: a phenotypic perspective. *Infection, Genetics and Evolution* 8(5), 737-746.

Martinez-Torres D., Chandre F., Williamson M. S., Darriet F.., Bergé J. B., Devonshire A. L., Guillet P., Pasteur N. and Pauron D. (1998). Molecular characterization of pyrethroid knockdown resistance (*kdr*) in the major malaria vector *Anopheles gambiae* s.s. *Insect Molecular Biology* 7, 179-184.

C1A

Norris, D. (2002). Genetic markers for study of the anopheline vectors of human malaria. *International Journal for Parasitology* 32, 1607-1615.

Noutcha M. A. E and C. I. Anumudu (2009). Entomological indices of *Anopheles gambiae* s.l. at a rural community in southwest Nigeria. *Journal of Vector-Borne Diseases* 46, 43-51.

Noutcha M. A. E and C. I. Anumudu (2010). Variations in species composition and infection rates in *Anopheles gambiae s.l.* across eco-vegetational zones in Nigeria and Cameroon. *Journal of Vector Borne Diseases* 47 (1), 51–54.

Nwagwu M., Anumudu C. I., Sodeinde O., Ologunde C. A., Obi T. U., Wirtz R. A., Gordon D. M. and Lyon J. A. (1998). Identification of a sub-population of immune Nigerian volunteers by antibodies to the circumsporozoite protein of *Plasmodium falciparum*. *American Journal of Tropical Medicine and Hygiene* 58, 684-692.

Onyabe D. Y. and Conn J. E. (2001). Genetic differentiation of the malaria vector *An. gambiae* across Nigeria suggests that selection limits gene flow. Heredity 87, 647-658.

Reimer L.J., Tripet F., Slotman M., Spielman A., Fonjo E., Lanzaro G.C. (2005). An unusual distribution of the *kdr* gene among populations of *Anopheles gambiae* on the island of Bioko, Equatorial Guinea. *Insect Molecular Biology* 14, 683-688.

Robert V. (2003). Une clé d'identification des anophèles: l'aile http://asmt.louis.free.fr/anopheles.html

Santolamazza F., Calzetta M., Etang J., Barrese E., Dia I., Caccone A., Donnelly M. J., Petrarca V., Simard F., Pinto J. and della Torre A. (2008). Distribution of *knock-down resistance* mutations in *Anopheles gambiae* molecular forms in west and west-central Africa. BioMed Central Malaria Journal 7, 74 http://www.malariajournal.com/content/7/1/74.

Scott J. A., Brongdon W. G. and Collins F. H. (1993). Identification of single specimen of the *Anopheles gambiae* complex by the polymerize chain reaction. *American Journal of Tropical Medicine and Hygiene* 49, 520-529.

Weill M., Chandre F., Brengues C., Manguin S., Akogbeto M., Pasteur N., Guillet P. and Raymond M. (2000). The kdr mutation occurs in the Mopti form of *Anopheles gambiae s.s.* through introgression. *Insect Molecular Biology* 11 (1), 11-19.

World Health Organisation (1999). TDR Progress Report 1997-1998. 14th programme report 51-52.

World Health Organisation (2005). The Work of the African Network on Vector Resistance to Insecticides, 2000-2004. 17*pp*.