Available online: www.notulaebiologicae.ro
Print ISSN 2067-3205; Electronic 2067-3264
Not Sci Biol, 2018, 10(1):97-101. DOI: 10.15835/nsb10110182
Original Article

A Simple Key for Identifying the Sibling Species of the Malaria Vector *Anopheles gambiae* (Giles) Complex by Polytene Chromosome Cytogenetics

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Abstract

It has been established that *Anopheles gambiae* complex sibling species are the major *Plasmodium* malaria vectors in Africa; however, not all the sibling species transmit the infection. Easier molecular methods, PCR-based assays, have been developed to distinguish the several members of the *A. gambiae* complex. However, malaria vector research in less developed countries, particularly sub-Saharan Africa, is being hampered by the lack of PCR facilities in laboratories and the cost of carrying out the assay within lack of funding. Hence, the present study was designed to develop a simple identification key, based on an affordable method of polytene chromosome cytotaxonomy, for identifying the major *P. falciparum* vectors. The Identification Key was successfully used to identify two members of the *A. gambiae* complex, *A. gambiae sensu stricto* and *A. arabiensis*, which are the most potent malaria vectors in Africa; even so, it could not be used to establish the infective and the refractory strains.

Keywords: *Anopheles arabiensis*, *A. gambiae*, cytotaxonomy, photomap, polytene chromosome, *sensu stricto*

Introduction

Malaria remains a major burden to human health in the sub-tropical and tropical regions of the world, where *Plamodium* malaria is the most prevalent and infectious (WHO, 1993; Hoffman *et al.*, 1996; Davidson, 2000; WHO, 2009). *Plasmodium* consists of four species, *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale* that cause human malaria (Nonstrand, 1978). Among the four species, *P. falciparum* is the most deadly (Hoffman *et al.*, 1996; Miller and Marley, 1999). Sub-Saharan Africa, however, suffers from the highest global malaria transmission levels, and hence, high mortality rate due to malaria (Fontenille and Simard, 2004; Hay *et al.*, 2005 and 2009); in Nigeria alone, ninety percent of malaria infections are caused by *P. falciparum*. *P. falciparum* is adapted to propagating alternately in *Anopheles* mosquitoes and human beings (Wikipedia, 2013).

The *A. gambiae* complex members are the dominant vectors of malaria in sub-Saharan Africa (Sinka *et al.*, 2010; Gregory and Yoosook, 2013). *A. gambiae* complex consists of eight sibling species, which include *A. gambiae sensu stricto* Giles; *A. bwambae* White; *A. merus* Dönitz; *A. arabiensis* Patton; *A. quadriannulatus* Theobald; *A. amharicus* Hunt, Coetzee and Fettene; *A. melas* Theobald; *A. comorensis* Brunhes, le Goff and Geoffroy (Gillies and DeMeillon, 1968; White, 1974 and 1985; Hunt *et al.*, 1998; Harbach, 2003; Coetzee *et al.*, 2013). Amongst these sibling species, *A. gambiae* and *A. Arabiensis* are the two major malaria vectors in Africa (Gillies and De Meillon 1968), particularly in Nigeria (Boreham *et al.*, 1979; Onyabe and Conn, 2001; Obembe and Awopetu, 2014). There are strains of *A. gambiae* complex sibling species in which the parasite cannot complete its life cycle; such strains are said to be refractory (Collins *et al.*, 1986; Chris *et al.*, 2011; Obembe and Awopetu, 2014).

Members of *A. gambiae* complex are morphologically identical, but genetically distinctive (Coluzzi *et al.*, 1979). Due to the genetic distinction, the sibling species can be distinguished by their polytene chromosome cytotaxonomy and molecular methods. The polytene chromosome cytotaxonomy is based on chromosomal inversions of the ovarian nurse cell polytene chromosomes (Coluzzi *et al.*, 1979; George *et al.*, 2010). Ten fixed inversions that can be used to differentiate the members of *A. gambiae* complex have been identified (Coluzzi *et al.*, 1979; Coluzzi *et al.*, 2002). In addition, drawings that depict the banding

Received: 09 Nov 2017. Received in revised form: 14 Mar 2018. Accepted: 22 Mar 2018. Published online: 27 Mar 2018.

patterns of *A. gambiae* s.l. polytene chromosomes are now available for characterizing the divisions and subdivisions of the species, as well as the locations of the inversions (Coluzzi *et al*., 2002; Holt *et al*., 2002).The molecular method is PCR-based and refers to species-specific single nucleotide polymorphisms (SNPs) in the intergenic spacer region (IGS) of the *A. gambiae* DNA (Scott *et al*., 1993; Fettene and Temu, 2003; Besansky *et al*., 2006; Wilkins *et al*., 2006).

The polytene chromosome cyto-taxonomic method is affordable, but it can only differentiate semi-gravid females, which constitute a small proportion of mosquito population in the wild; molecular method is easier and it can identify all stages and genders in the population. Nevertheless, in sub-Saharan Africa, particularly in Nigeria, the use of molecular method in identifying the members of malaria vector species complexes is restricted by lack of the PCR facilities in laboratories and the cost of carrying out the assay coupled with lack of research funding; consequently, malaria vector research is restricted. Hence, there is need for a simple identification key based on the affordable polytene chromosome cytotaxonomy so as to encourage malaria vector research in Africa.

Materials and Methods

Study area

Two selected areas in Osun State, Ilesa (4°44′E, 7°37′N) and Ile-Ife (7°28′N, 4°34′E), in South-Western Nigeria were sampled. *Anopheles* larvae were collected from stagnant pools of water in these locations and transported to the laboratory at Obafemi Awolowo University, Ile-Ife, Nigeria. The *Anopheles* larvae were identified by using their resting position in water, as well as a diagnostic feature, lack of a respiratory siphon but spiracles on the 8th abdominal segment.

Rearing F1 generations of Anopheles mosquitoes

The *Anopheles* larvae collected from the study areas were used to breed F_1 colonies of *Anopheles* larvae in the laboratory. The F_1 *Anopheles* larvae species were validated by observing the diagnostic features, resting position of the larvae in water, absence of breathing siphon and presence of palmate hairs on the eighth and fourth abdominal segments respectively, under a dissecting microscope (WHO, 1997; Mark *et al*., 2007). The validated F_1 *Anopheles* larvae were fed with biscuit diet until they emerged as adults. Gender grouping of the F_1 adult mosquitoes was carried out on the third day of emergence based on their diagnostic features, antennae and palps structures (Service, 1980; WHO, 1997).

Separation of female Anopheles gambiae from the stock

The F_1 female *Anopheles* mosquitoes were anaesthetized on the fourth day and *A. gambiae* females were selected from the stock using a standard identification key (Mark *et al*., 2007). Further species authentications were carried out by using the collated Identification Key from Hamon and Adam (1963), De Meillon (1947) and Evans (1938). The female *A. gambiae* mosquitoes that were identified were infected with *P. falciparum*.

Female A. gambiae infection

In preparation for the infection experiment, the F_1 female mosquitoes were starved for 18 to 20 hours. On the day-5, after emergence, the female mosquitoes were allowed to feed on a pre-diagnosed *P. falciparum* infected individual for about 15 minutes such that the mosquitoes became engorged. Mosquitoes that did not feed on the infected individual, as well as the ones that were not engorged, were sacrificed in chloroform. The infected mosquito colonies were kept on 10% sugar solution regimen in the laboratory with ambient temperature, between 26 °C and 32 °C. They were maintained in the laboratory for 16 to 18 days after infection, such that *P. falciparum* would have reached their salivary glands (WHO, 1975).

Identification of the A. gambiae sibling species

Sibling species identification was carried out by using a simple Identification Key developed from Coluzzi *et al*. (2002) and George *et al*. (2010) (Table 1). The infected mosquitoes were deprived of food for 18 to 20 hours after which they were fed with blood a day before the dissection. Half gravid females (Clements, 1992) were dissected between 20 - 24 hours after blood feeding for ovarian extraction. Stained polytene chromosome spreads were prepared from fixed ovaries (Cornel, 2007). Images of the polytene chromosome spreads that showed suitable levels of polytenization were obtained by using the digital imaging system of the Accuscope 3000 LED phase contrast microscope (Accuscope Inc., 2011). The chromosome images were processed by the Macormedia Fireworks image editing software version 8.0 (Macromedia Inc., 2005). Chromosome arm recognition was carried out by using standard arm recognition landmarks (George *et al*., 2010). With reference to Coluzzi *et al*. (2002) and George *et al*. (2010), 2R arm cytogenetic maps of the mosquito polytene chromosomes were developed and the inversion (2R*j*, 2R*b*, 2R*c*, 2R*u* and 2R*d*) breakpoints were characterized. The Identification Key in Table 1 was successfully used for identifying the sibling species of the mosquitoes.

Table 1. Simple identification key for identifying the sibling species of *A. gambiae* complex using common polymorphic inversions

Inversion	*A. gambiae*	*A. arabiensis*	*A. bwambae*	*A. melas*	*A. merus*	*A. quadriannulatus*
2R*j*	+	-	-	-	-	-
2R*b*	+	+	-	-	-	-
2R*c*	+	+	-	-	-	-
2R*u*	+	-	-	-	-	-
2R*d*	+	-	-	-	-	-

+ = present, - = absent

The infective and the refractory strains

The mosquito salivary glands were dissected immediately after ovary extraction. A drop of physiological solution (Hayes, 1953) and a cover slip were then placed on the salivary glands. They were broken by a gentle pressure applied to them in order to free the sporozoites into the physiological solution. The glands were then inspected under x40 objective of microscope for infection by *P. falciparum*.

Results

P. falciparum sporozoites were observed in the salivary glands of the mosquitoes, which indicated that the mosquitoes were susceptible to *P. falciparum* infection, and hence, they were infective. However, the salivary glands of some of the mosquitoes did not bear sporozoites, indicating that those mosquitoes were refractory to *P. falciparum* infection. Infective and the refractory strains were recorded in both locations.

Proportions of the infective and the refractory strains

Table 2 shows the proportions of the infective and the refractory strains of the *A. gambiae* mosquitoes from the two study areas. There was a significant difference between the proportions of infective and refractory strains within each study area ($P < 0.05$); contrarily, the proportions of refractory strains with the proportions of infective strains were not significantly different between the two study areas ($P > 0.05$).

Sporozoite rate

Sporozoite rates of the mosquitoes from both study areas are shown in Table 3. The sporozoite rates between the study areas were not significantly different ($P > 0.05$).

Sibling species identification

The Identification Key (Table 1) was successfully used to identify two sibling species, *A. gambiae sensu stricto* and *A. arabiensis*, of the *A. gambiae* complex. The observed infective strains include the two members of the complex, *A. gambiae sensu stricto* and *A. arabiensis*. It was noted that *A. gambiae sensu stricto* was the only refractory strain recorded. The infective strain and the refractory strain of *A. gambiae sensu stricto* have the same banding patterns, and hence, they could not be distinguished by the Identification Key.

Discussion

There is insufficient information on the identification of the sibling species of malaria vectors and their infective and refractory strains in south-western Nigeria (Obembe and Awopetu, 2014), perhaps, due to lack of facilities and funding for carrying out the molecular, PCR-based assay, method of identification. Hence, the current study was designed to promote malaria vector research, not only in Nigeria, but also in other less developed countries of the world, particularly in sub-Saharan Africa.

The proportions of *A. gambiae* complex recorded in the study areas were high, which shows that the members of *A. gambiae* complex are greatly susceptible to *P. falciparum* infection. In a similar research by Obembe and Awopetu (2014) in the study area, virtually, the same results were obtained. Similar reports were also recorded by Collins *et al*. (1986) and Alvaro *et al*. (2012). This corroborates the idea that the parasites are capable of evading the mosquito immune responses (Osta *et al*., 2004). However, the fact the parasite was not successful in reaching the salivary glands of some of the mosquito's supports the reports by Julián (2010) and Chris *et al*. (2011) that the mosquito inherent defence system is capable of terminating the development of all the parasites before they reach the salivary glands leading to total refractoriness. It also confirms the report by Obembe and Awopetu (2014) that refractory strains of *A. gambiae* are present in South-Western Nigeria, though at a very low frequency.

However, the sporozoite infection rates that were obtained were significantly higher than the ones previously reported, 6.70% and 6.30% in 2001 and 2002 respectively, at Igbo-Ora, Oyo State in South-Western Nigeria (Noutcha and Anumdu, 2009), as well as at Badagry Axis of Lagos Lagoon, Lagos State, Nigeria (Oyewole *et al*., 2010).

Table 2. Proportions of refractory (R) and infective (I) *A. gambiae* mosquitoes from the study areas

Study Area	a	b	c	d	e
	Total number of mosquitoes dissected	Number of R	Number of I	Proportion of R (b/a)	Proportion of I (c/a)
Ilesa	58	12	46	0.207	0.793
Ile-Ife	62	11	51	0.178	0.823
Overall	120	23	97	0.192	0.808

R = Refractory mosquitoes; I = Infective mosquitoes

Table 3. Sporozoite infection rates of *A. gambiae* mosquitoes from the study areas

Study Area	a	b	c
	Total number of mosquitoes dissected	Number of infective mosquitoes	Sporozoite infection rate (%) (b/a x 100)
Ilesa	58	46	79.31
Ile-Ife	62	51	82.23
Overall	120	97	80.83

However, Obembe and Awopetu (2014) has reported similarly high *P. falciparum* sporozoite rates in the study areas. Okwa *et al*. (2006) also reported a high sporozoite rate (62.9%) for *A. gambiae* in Badagry area of Lagos, Nigeria. In a related study, 83.52% *P. falciparum* sporozoite infection rate was recorded for *A. gambiae* (Ndiath *et al*., 2011). The high *P. falciparum* sporozoite rates shows that the *A. gambiae* complex members are prominent and potent *P. falciparum* vectors in South-Western Nigeria and similar report was given by Gilles and Coetzee (1987), Annon (2003), Okwa *et al*. (2007) and Okwa *et al*. (2008).

The identified members of the *A. gambiae* complex, *A. gambiae sensu stricto* and *A. arabiensis*, as similarly reported by Okwa *et al*. (2008) and Obembe and Awopetu (2014), are the major *P. falciparum* vectors in South-Western Nigeria.

Conclusions

The present study showed that *A. gambiae sensu stricto* and *A. arabiensis* are potent malaria vectors in south-western, Nigeria. In addition, they can be identified by the Identification Key based on the affordable ovarian polytene chromosome cytotaxonomy. However, the method could not be used to characterize the refractory and infective strains of the *A. gambiae sensu stricto*.

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