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Anopheles Species Diversity and Breeding Habitat Distribution and the Prospect for Focused Malaria Control in the Western Highlands of Kenya

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Abstract: Malaria in the western highlands of Kenya is unpredictable and occurs in form of severe outbreaks and epidemics resulting in loss of life and exerts a strain on public health services. This underlies the continued need for better understanding of the disease determinants to formulate specific and focused intervention strategies. A 1 year study was undertaken in two study sites in Nandi district to determine *Anopheles* species diversity, abundance and breeding habitat distribution. Indoor and outdoor adult Anopheles mosquitoes were collected biweekly from randomly selected houses by pyrethrum spray capture method and light traps, respectively. Anopheles larval stages were collected from breeding sites by standard dippers and reared into adults under laboratory conditions. Adult *Anopheles* species were identified based on morphological features and sibling species by Polymerase Chain Reaction (PCR). *Anopheles gambiae sensu lato* was the most prevalent known vector contributing 95.4%, *An. funestus* and *An. arabiensis* each 2.3%. All *An.gambiae s.l* samples were identified by PCR as *An. gambiae sensu stricto* suggesting that this could be the only sibling species of the *An. gambiae* complex present in study sites. The epidemiological and public health significance of known malaria vectors and non-vector anopheles species is discussed.

Key words: Breeding habitats/sites, malaria vector, sibling species, polymerase chain reaction, transmission, Kenya

INTRODUCTION

Malaria in the western highlands of Kenya is unpredictable disease with increasing frequency and intensity in transmission. The establishment and spread of the disease in this highland area depends on the presence of and relationship of several epidemiological factors the most important being, the host, the agent and the environment. Man is the vertebrate host and the Anopheles mosquito in the agent of transmission/vector whereas the Plasmodium parasite is the causative agent of malaria.

Malaria vector and non-vector species population structure and density in any locality is not static (Duffy, 1977). Malaria vector (s) and non-vectors may periodically extend their range beyond their normal area of distribution when temporary suitable conditions occur in neighboring areas (WHO, 2010). Most *Anopheles* species are known to change their ecological range, behavior by adapting to new climatic, ecological and human induced changes (Muriu *et al*., 2008). This may not be frequent but it does occur particularly in this era of global warming and may result in serious public health implications. Human activities particularly related to land-use evident in the study area could promote changes in vector and non-vector species diversity and malaria transmission in future in several ways as reported elsewhere (Walsh *et al*., 1993; Patz *et al*., 2000). For instance man-made activities such as swamp reclamation and brick making creates more human-made aquatic habitats for the *Anopheles* species. Increased cattle grazing create more open habitats with elevated temperature that favor faster Anopheles larval development hence increased adult density and possible malaria transmission (Minakawa *et al*., 2004). Man-made environmental changes may also have a bearing on the diversity of the vectorial systems of an area and subsequently result in eco-epidemiological stratification and invasion of area by new vectors. Human activities and topography could also have an effect on the diversity and distribution of disease vector breeding habitats. Therefore, we hypothesized that human activities, environmental changes and regular travel to and from the

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neighboring Lake Victoria lowlands could result in changes in *Anopheles* species composition which may have implications on transmission, epidemiology and control of not only of malaria but also other vector-borne diseases. The purpose of the study was to assess the *Anopheles* species diversity and locate and identify breeding habitats in two sites in a malaria epidemic-prone area in the western highlands of Kenya.

MATERIALS AND METHODS

Study sites: The study was conducted in North Nandi District [0°21'52 N and 0°16'56 N in longitude and 35°5'20°E and 34°59'7 E in latitude] in the highland areas of Kipsamoite and Kapsisiywa each with 7 and 10 villages, respectively. The study sites were selected because, they were located 1500 m above sea level, an altitude defined as characterizing the highland area and malaria epidemics and outbreaks had been reported within the sites previously.

The topography of the study sites comprises hills, valleys and plateaus. Rivers and streams run along the valley bottoms in the valley ecosystem and reclaimed and natural swamps are a common feature. The study area has two rainy seasons, long rains season from March to May, referred to long rains on the account of duration and high amount of rainfall received in many parts of the highlands. The second season is the short rains from the months of October to December during which period, the area experiences depressed rainfall that is also poorly distributed both in space and time. There are variations in temperature the warmest temperatures are experienced in March and the coldest in July with the mean monthly temperatures ranging from 17-19248°C.

***Anopheles* species sampling points:** Anopheles mosquito samples were collected from January to December 2008 in a total of 17 villages within Kipsamoite and Kapsisiywa study sites. The 40% (n = 656) of the households were randomly selected, coded and used as sampling points distributed as follows: Kipsamoite with a total of 666 households out of which 265 (40%) were sampled; Kapsisiywa with a total of 982 out of which 391 (40%) were sampled.

***Anopheles* species breeding habitat identification:** Mosquitoes are capable of colonizing just about very conceivable type of water body except fast running water in rivers and streams. Systematic ground surveys were conducted at 2 weeks intervals in months of January to December representing the dry and rainy season to determine the possible Anopheles aquatic breeding habitats. This excluded fast running water because mosquitoes breed in calm, slow, moving water and water in containers in houses because it was meant for household use and tree holes because of non accessibility. The following aquatic habitats were found present in the study sites and constituted potential breeding sites: pools and puddles, foot/hoof prints, drains and ditches, streams and river edges, ponds, natural/ disturbed swamps and marshes, bore holes, wells and springs. The occurrence of anopheline larvae in each habitat was determined by using a standard dipper for large habitats (>0.5 m^2) for smaller aquatic habitats (<0.5 m^2) like foot/hoof prints a small sieve and pipette were used to scoop water.

A dipper (13 cm in diameter and 6.5 cm deep) with a handle)/sieve/pipette was gently lowered at an angle of 45°C just below the water surface so that water flowed into it or sucked (by pipette) together with any larvae that might be present. During dipping, care was taken not to disturb water too much and make larvae swim downwards. The filled dipper/sieve/pipette was carefully lifted, taking care not to spill the water containing the larvae. The drawn water containing the larvae was poured into white rectangular trays and checked visually and larval samples when present were collected by pipette. Ten dipper/pipette/sieve collections were made per each aquatic habitat. If none had anopheline larvae then the site was declared anopheline negative. The larval samples from each habitat were transferred into labeled (with date, collection site and type of habitat) vials and transported to Kenya Medical Research Institute (KEMRI) for rearing into adults and subsequent identification of adults. The identified anopheline positive breeding sites were noted, location recorded, counted and categorized as Anopheles larval collection points for outdoor collections. The results of the rainy and dry season breeding habitat number are shown in Table 1.

Collection of outdoor larvae and adult *Anopheles* species: Anopheles larval collection from identified breeding sites was carried out every 2 weeks from 0600-0900 h. The collected larval samples were packet in cool-box and transported to the Kenya Medical Research Institute (KEMRI) laboratory and reared to adults under the following conditions: temperature 27°C, 80% relative humidity and 12:12 light: dark schedule and brewer's yeast as food. Upon emergence from pupa, the adults were identified by morphological features using identification keys. Out-door adult Anopheles mosquitoes in animal shelters were collected by use of Centers for Disease Control (CDC) miniature light traps (J.W. Hock Ltd, Gainsville, Fl., USA).

Table 1: Rainy and dry season Anopheles identified breeding sites

	Rainy season		Dry season	
Survey number	No. of breeding habitats (%)	No. of anopheline positive habitats (%)	No. of potential breeding habitats (%)	No. of anopheline positive habitats (%)
1	40 (23.0)	15 (20.8)	27 (30.0)	9 (25.7)
2	36 (21.3)	14 (19.4)	18 (20.0)	7 (20.0)
3	45 (26.6)	23 (32.0)	21 (23.3)	9 (25.7)
4	48 (28.4)	20 (27.8)	24 (26.7)	10 (28.6)
Total	169 (100.0)	72 (100.0)	90 (100.0)	35 (100.0)

Table 2: Anopheles diversity and abundance

Anopheles species	Number collected indoors and outdoors)	Relative abundance (%)
**An. gambiae s.s.*	41	10.6
**An. funestus*	01	0.3
**An. arabiensis*	01	0.3
+*An. christyi* (Newstead and Carter)	108	27.9
An. demeilloni (Evans)	94	24.3
An. coustani (Levaran)	66	17.1
An. squamosus (Theobald)	51	13.0
An. harperi (Evans)	21	5.4
An. leesoni (Evans)	02	0.5
An. longipalpis (Theobald)	01	0.3
An. ziemanni (Grunberg)	01	0.3
Total	387	100.0

*Known human malaria vectors; +Ancestor of all human malaria vectors (Anthony *et al*., 1999)

Collection of indoor adult *Anopheles* species and identification: Total indoor resting mosquitoes were collected from randomly selected households by the pyrethrum space spray method also called Pyrethrum Spray Collection/Catches [PSC] method (WHO, 1975) every fortnight. White sheets were spread on the floor and all the windows, doors and all other exit points closed. Pyrethrum extract [0.2% in kerosene] was sprayed on all eaves, doors, windows and in the entire space of all rooms in the house and the house closed for 10-15 min. All knocked down *Anopheles* species were collected carefully with the forceps and placed in petri dishes lined with moist filter paper. The collections were transported to KEMRI laboratories preservation on silica gel in Eppendorf tubes prior to species identification.

All the anopheles mosquito collections were sorted out to separate the females from males. The females were identified to species level using morphological features with the aid of identification manuals (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987) and Anopheles identification soft ware CD. The results are shown in Table 2.

The females of all morphologically identified female *Anopheles gambiae s.l.* mosquitoes collected from houses and those reared to adults from larval mosquitoes were identified to sibling species using PCR as described by Scott *et al.* (1993). The PCR assay involved the following key steps: mosquito DNA extraction using the potassium acetate precipitation technique making of PCR master mix [mixture of buffer, ions, primers and enzymes in water];

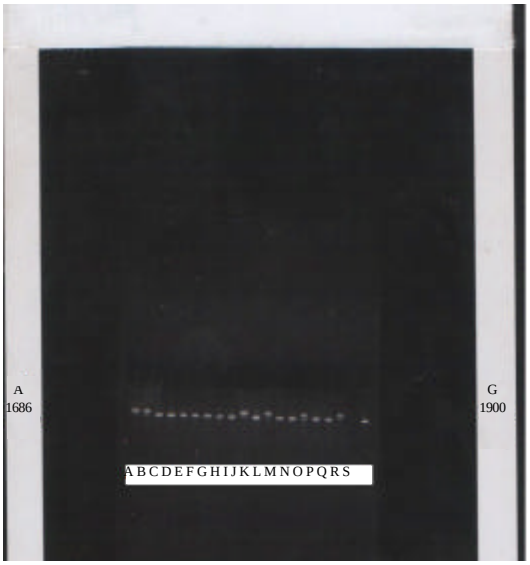

Fig. 1: PCR comparative band distribution of *An. gambiae s.s* and *An. arabiensis*. Sample A, B, J, L. O and R were *An. arabiensis* controls. Sample S was *An. gambiae s.s* control; Samples C, D, E, F, G, H, I, K, M, N, P, Q were *An. gambiae* confirmed as *An. gambiae s.s*. Assay conditions: 3% Agarose, 200 V, 149 mA, 029, 20 min

electrophoresis; gel visualization and photography. Samples not identified after 3 PCR reactions were marked as unknown. The distribution of bands in the gel after electrophoresis was used to identify and determine *Anopheles gambiae* siblings as *An. gambiae s.s.* and *An. arabiensis* species whose oligo primers had been included in the master mix as controls. The results are shown in Fig. 1.

RESULTS

Anopheles larval habitat types: Most of the larval habitats were man-made that is arise from human related activities and confined to valley bottoms. The area has experienced extensive swamp reclamation for crop production and creation of tree nurseries. The drainage channels formed

water collection points in both dry and wet season. Bore holes common in tree nurseries and homes had water throughout the year. Hoof/foot prints in drained swamps, pastures were important breeding sites during dry season. Natural springs, undisturbed swamps, marshes were important permanent breeding sites particularly sunlit edges with less vegetation. In rivers and streams, breeding was common on the edges where water flow was slow and calm. During heavy rains when most of the channels become fast flowing streams/rivers, the Anopheles shifted breeding to water collections in cattle hoof prints in the reclaimed swamps. Brick making was a common economic activity that created suitable mosquito breeding grounds. Brick making resulted in innumerable borrow pits (flooded depressions left by soil excavation for brick material) provided prolific breeding sites for vectors. Most of the breeding sites easily dried up in a few days of dry spell, a few become more confined towards the valley bottoms and reappeared after rainfall while others were easily washed out (flush-out effect) following heavy downpour.

A variety of anopheline larval habitats identified in the study area were rivers, streams, boreholes, springs, swamps and animal hoof-prints that were more open to sunlight and plenty of algae. A majority of them were categorized as man-made. During the rainy season, 169 aquatic habitats were identified as potential breeding habitats and anopheline larvae were found in 72 (43%) habitats. In the dry season, the potential breeding habitats identified were 90 and 39% of them were anopheline positive larval habitats (Table 1). Man-made habitats for instance depressions associated with brick-making supported Anopheles mosquitoes in overcoming the dry period in many areas. These and other man-made habitats played a role in maintaining breeding throughout the year. Results of the ground survey showed that the number of potential anopheline aquatic habitats increased 1.9 fold during the rainy season over the dry season (169 vs. 90). However, there was no significant difference (ANOVA, $p = 0.436$) between the number of anopheline-positive breeding habitats in the rainy and in the dry season. Anopheline larval habitats were clustered at valley bottoms and composed of mainly man-made habitats.

***Anopheles* species diversity and abundance:** A total of 387 female adult Anopheles belonging to 11 species collected from study area. They were identified by their morphological features and categorized in non-vectors and known malaria vectors. The non-vectors belonged to 8 species comprising of *An. christyi* Newstead and Carter, the most predominant species and ancestor of all malaria vectors (Anthony *et al*., 1999) followed by *An. demeilloni* Evans, *An. coustani* Levaran, *An. squamosus* Theobald and *An. harperi* Evans. Other species found in low numbers were: *An. ziemanni* Grunberg, *An. leesoni* Evans and *An. longipalpis* Theobald. The known human malaria vectors were *An. gambiae*, *An. funestus* and *An. arabiensis* comprising 11% in 3 species. *An. gambiae s.l.* was the most predominant known malaria vector species while the other two species were rare. All known malaria vectors were collected from indoors an indication of their close association with human habitation. The diversity, relative abundance of vector and non-vector Anopheles mosquitoes in the study area is shown in Table 2.

PCR assay of anopheles gambiae: The 41 *An. gambiae* sample specimens collected from the study sites were analyzed by Polymerase Chain Reaction (PCR). All were found to belong to one sibling species *An. gambiae sensu stricto (s.s)* indicating that it was possibly the only sibling species from the *An. gambiae* complex circulation in the study area. For quality assurance and comparative purposes, a run containing both *An. gambiae* and *An. arabiensis* was done to show how the results would have been in case the other sibling species *Anopheles arabiensis* would have been present in the samples. In both cases, single bands were visualized at different levels and photographed for *An. arabiensis* and *An. gambiae s.s*. The results are presented in form of photographed gel under UV light in (Fig. 1).

DISCUSSION

The study area is characterized by hill-valley topography. These topographical features determine the formation of aquatic habitats. Hill-valley topography facilitates run-off down-hill to settle in the valley bottoms forming aquatic habitats such as springs, streams, rivers and swamps. The aquatic habitats surveyed indicated that Anopheles breeding sites were confined at the valley bottoms mainly in temporary, man-made habitats. It is suggested that probably *Anopheles* species prefer open sunlit man-made habitats in which predation on larvae is less prevalent in temporary habitats than it is in large permanent habitats and competition for resources is less common in newly created man-made habitats (Service *et al*., 1977; Washburn, 1995). In related studies, Minakawa *et al*. (2004) reported a similar *An. gambiae s.s* larval habitat distribution in a highland site in Kenya. The locations of breeding sites at valley bottoms suggest that these locations could be associated with higher risk of

malaria because they are more likely to become malaria hot spots (transmission focal points) (Ernst *et al*., 2006). Therefore, communities living near valleys have a higher risk of contracting disease than those living uphill (Lindblade *et al*., 2000; Balls *et al*., 2004). This is in contrast to the lowland area of Lake Victoria Basin which is generally a flat terrain and Anopheles breeding habitats are many and widely dispersed (Gimnig *et al*., 2001; Minakawa *et al*., 2002a, b). These generate high malaria vector densities throughout the year hence perennial malaria transmission in the lowlands (Bodker *et al*., 2003) compared to seasonal transmission in the highlands.

The finding that larval habitat distribution was aggregated and mostly man-made suggested that larval control in the study area could be targeted to the aquatic habitats at the valley bottoms. Githeko *et al.* (2006) suggested that in such situation, effective vector control could target the confined breeding sites at specific sites and could also involve community participation. Targeted larval control in the study area would be even more effective if undertaken during the dry season just before the onset of rainy season when breeding habitat distribution is limited by high evaporation rate leading to drying of most of the aquatic habitats. And the few that remained were more confined towards valley bottoms. It is suggested that source reduction (larval control) is the method of choice for mosquito control when mosquito species targeted are concentrated in small number of discrete habitats as is the case in the study sites. However, in severe epidemic situations as is common in the western highlands of Kenya, it would be prudent to apply Integrated Vector Management (IVM). In this regard, source reduction (Githeko *et al*., 2006) and targeted IRS (Chrispinus *et al*., 2011) would be appropriate control strategies. Source reduction has been one of the important malaria measures used to suppress malaria in Brazil (Soper and Wilson, 1943), Tanzania (Bang *et al*., 1975, 1977) and United States, Israel, Italy (Kitron and Spielman, 1989).

Human activities particularly related to land-use evident in the study area are likely to promote malaria transmission in future in several ways as reported elsewhere (Walsh *et al*., 1993; Patz *et al*., 2000). For instance swamp reclamation creates more man-made aquatic habitats for the local *An. gambiae s.s*, the predominant malaria vector in the area. Increased cattle grazing creates more open habitats with elevated temperature that favor faster vector larval development hence increased adult vector density and malaria transmission (Minakawa *et al*., 1999, 2004). Higher adult densities of *Anopheles gambiae* complex have been reported in houses near established malaria vector breeding habits in some highland areas (John *et al*., 2004) and people living near valley bottoms (near to breeding habitats) are likely have more vector-human contact than those up-hill and therefore more malaria cases.

Entomological results indicate the presence of both known malaria vectors and non-vector species in the study area. The significance of *Anopheles* species not known to act as vectors is not clear. However, it is possible that some of the species present a nuisance of mosquito-bites rather than transmit malaria (Koenraadt, 2003). The study sites were characterized with large herds of livestock (cattle, sheep and goats) that were often kept near/around human habitations. For the zoophilic/ antropophilic species in this group (including *An. christy*, *An. demillon*, *An. harperi*, *An. leesoni* and *An. longipalpis)*, the initial attraction emanating from cattle/goats/sheep kept inside or around human houses may influence their feeding behavior (Oyewole *et al*., 2007). As such a possible change in behavior in host seeking Anopheles may increase the risk of man becoming a regular source of blood meal by both zoophilic and anthropophilic species. This could create a close link between man-animal-mosquito favorable for the transmission of other mosquito-borne diseases in animals and man. For instance, *An. coustani* laveran widespread and abundant over much of the African continent also encountered in the study area readily feeds on humans outdoors (Coetzee, 1983) and play a role in the transmission of disease arboviruses (Logan *et al*., 1991; Gordon *et al*., 1992; Coetzee, 1994). Other non malaria vector species of the *gambiae* and *funestus* complexes are known to transmit *O'nyong nyong* virus in East Africa (Williams *et al*., 1965). It is possible that some of the non-vector anopheles species may be of local importance in disease transmission as incidental vectors as reported elsewhere (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). In this regard the non-malaria vectors should not be ignored. Efforts to eliminate them could turn out to be a community motivation for adopting malaria control and prevention methods in the present study area where adoption of malaria control measures is low because of the sporadic nature of the disease.

The known malaria vectors present in study area were *Anopheles gambiae*, *An. funestus* and *An. Arabiensis*. The three *Anopheles* species are known to be the most efficient malaria vectors in the world (Besansky, 1999). This is because of their marked preference for human environments and for humans as hosts and due to their

rapid adaptation to changes in the environment induced by human habitation and agricultural development (Collins and Besansky, 1994; Powell *et al*., 1999). *An. gambiae* was abundant malaria vector and the most prevalent of the three species in the present study. These findings were consistence with related studies in the same or neighboring sites as well as other highland regions in Africa (Collins *et al*., 1988; Petrarca *et al*., 1991). Previous vector studies in Nandi indicated malaria vector composition of 98% *An. gambiae* complex and 2% *An. funestus* (Roberts, 1964; Ernst *et al*., 2006). In related studies in many parts of Africa, *An. gambiae* is found together with the equally important vector *An. funestus* (Charlwood *et al*., 2003; Nkuo-Akenji *et al*., 2006). *An. funestus* and *An. arabiensis* were rarely encountered with only one specimen of each collected translating into 2.4% of the total malaria vectors. This could be because the two species have difficulties/apparent failure to colonize high altitudes successfully. However, there is need for extensive studies on larval and adult surveys and dispersal experiments targeting these species to come up with a clear picture on diversity in the study area.

Malaria vector (s) and non-vectors may periodically extend their range beyond their normal area of distribution when temporarily suitable conditions occur in neighboring areas (WHO, 1998). This may explain the presence of *An. ziemanni* in study area which is widely encountered in West Africa extending to Ethiopia with scanty, localized distribution in East Africa (Gillies and De Meillon, 1968). In Kenya, it has been previously reported mainly in the low lands of Lake Victoria basin (Khamala, 1971; Kamau *et al*., 2006). In the present study, the species was encountered but its vectorial importance is not clear although, it is known to be susceptible to and can maintain malaria parasites (Gillies and De Meillon, 1968), feeds on both man and animals (Kamau *et al*., 2006). The species may be undergoing a phase of adaptation to live in highland areas in proximity to the normal habitat, the lowlands. There is need for further field and experimental studies on *An. ziemanni* as regards possible role in malaria transmission in both low and high altitude areas. The presence of a species of well known malaria vector, *An. arabiensis*, a predominant vector in lowlands at high altitude raises curiosity. If determined and confirmed in larger long-term studies, the presence of *An. arabiensis* at the present high altitude area would support scanty reports that the species is capable of breeding and transmitting malaria in a highland area (Chen *et al*., 2006). It is possible that regular travel between Lake Victoria lowlands and the western highlands could introduce the vector into highlands. Both *An. gambiae s.s* and *An. arabiensis* have similar requirements for their larval environment (Service *et al*., 1977; Gimnig *et al*., 2001). Therefore, there is a possibility that *An. arabiensis* imported into the highlands could become established and become an important malaria vector together with *An. gambiae s.s* in future. Whenever these two species occur together, populations of *An. arabiensis* are known to survive the dry season better while populations of *An. gambiae s.s.* peak shortly after onset of rainy season (Koenraadt, 2003). If this scenario is established, then malaria transmission in the western highlands of Kenya could become perennial as opposed to the current seasonal transmission.

CONCLUSION

Malaria transmission in the western highlands of Kenya is seasonal, focal in nature, sporadic with more cases reported in communities living at valley bottoms where highest concentration of breeding habitats are located. It is concluded that intervention strategies should therefore be appropriately focused at these points when identified for cost effectiveness and better results.

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