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Full Length Research Paper

Diversity of *Anopheles* species and prevalence of malaria in a highland area of Western Kenya

Mulambalah Chrispinus S.[1]*, Ngeiywa Moses M.[2], Siamba, Donald. N[1] and Vulule John M.[3]

[1]Department of Biological Sciences, Masinde Muliro University of Science and Technology, P. O. Box 190 Postcode 50100, Kakamega, Kenya.
[2]Department of Biological Sciences, Moi University, P. O. Box 1125, Eldoret, Kenya.
[3]Kenya Medical Research Institute, P.O. Box 1578 Postcode 40100, Kisumu, Kenya.

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In the western highlands of Kenya malaria is an unpredictable disease with increasing frequency and intensity of transmission. Sometimes the disease occurs in form of severe outbreaks and epidemics that result in loss of human life and exerts a strain on public health services. This underscores the continued need for better understanding of the determinants of transmission of the disease in order to formulate specific and focused intervention strategies. A one year study was undertaken in two study sites in Nandi district in the western highlands of Kenya to determine diversity and abundance of *Anopheles* species, and parasite species most associated with the disease. Adult *Anopheles* mosquitoes were sampled biweekly from randomly selected houses by pyrethrum spray capture method and light traps. Larval mosquitoes were sampled from breeding sites by standard dippers and reared into adult stages under laboratory conditions. Adult *Anopheles* species were identified based on morphological features and sibling species by polymerase chain reaction (PCR). Microscopic examination of blood smears was used to confirm malaria infection, to identify *Plasmodium* species and determine species prevalence. *Anopheles gambiae* s.l was the most prevalent known vector contributing 95.4%, with *Anopheles funestus* and *Anopheles arabiensis* 2.3% each. All *Anopheles gambiae* s.l samples were identified as *A. gambiae s. s.* suggesting that this could be the only sibling species of the *A. gambiae* complex present in study area. *Plasmodium falciparum* was the most prevalent (90%) while *Plasmodium malariae* was 10%. There was significant difference in the malaria parasite species prevalence (χ^2 P<0.05) but there was no significant difference in the parasite species prevalence between the study sites (χ^2 P>0.05). The epidemiological significance of known malaria vectors and non-vector anopheles species is discussed.

Key words: Malaria, prevalence, anopheles, sibling species.

INTRODUCTION

Malaria is a mosquito-borne infectious disease caused by protozoa of the genus *Plasmodium.* The disease is wide spread in the tropical and sub-tropical regions of the world including parts of the Americas, Asia and Africa (Snow et al., 2005). The establishment and spread of malaria within a geographical area can vary greatly between villages and households (Greenwood et al., 1989; Gamage-Mendis et al., 1991). In the western highlands of Kenya, malaria transmission is related to the seasons and population movements (Otsyula, 2002) and occurs in form of epidemics (Hay et al., 2002a) with high morbidity and mortality rates. Malaria epidemics in the western highlands are unpredictable, focal in nature with transmission varying from locality to another (Kacey et

*Corresponding author. E-mail: csmulambalah@gmail.com. Tel: +254721347269.

Abbreviations: KEMRI, Kenya medical research institute; **CDC,** Centers for Disease Control; **PSC,** pyrethrum spray collection/catches; **PCR,** polymerase chain reaction; **DNA,** deoxyribonucleic acid; **UV,** ultra violet.

al., 2006). *Plasmodium falciparum* has been the parasite species consistently associated with malaria outbreaks in the western highlands of Kenya (Some, 1994; Githeko and Ndegwa, 2001; Hay et al., 2002b). However, it is not clearly known whether other *Plasmodium* species played any role in malaria outbreaks and epidemics in these highland areas. Malaria vector and non-vector *Anopheles* species population structure in any locality is not static. Both vector(s) and non-vectors may periodically extend their range beyond their normal area of distribution (WHO, 1998). In the highland areas of Kenya, *Anopheles* species are known to change their ecological range, behavior, by adapting to new climatic, ecological and human induced changes (Simon et al., 2008).

This may not be frequent but it does occur particularly in this era of global warming and varied land use practices, and may have serious public health implications and on the epidemiology of malaria. Human induced activities have a strong association with the malaria vectors in the Kenyan highlands particularly as regards *Anopheles gambiae* larval development and adult density (Minakawa et al., 2004); enhanced survivorship and reproductive fitness of adult *A. gambiae* (Afrane et al., 2006) and creation of ecological factors and habitats suitable for *A. gambiae* breeding (Kacey et al., 2006; Goufa et al., 2007). Man-made environmental changes therefore can have a limiting and or enhancing factor on the diversity of the vectorial system of an area and invasion by new vectors. Previous studies in the western highlands indicate that *A. gambiae* as the primary malaria vector with *Anopheles. funestus* as a secondary vector (Snow et al., 1999; Koenraadt, 2003; Kacey et al., 2006). These findings however do not specify which sibling species of *A. gambiae* complex was involved in malaria transmission in the western highlands. This provided an opportunity to investigate the *Anopheles* species composition with regard not only to malaria transmission but also of other mosquito-borne infections. The purpose of the study was to assess the *Anopheles* species diversity and *Plasmodium* species prevalence at two study 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. Also, two government health centers present within the sites were considered important in carrying out malaria parasite prevalence studies because they served large and varying catchment populations and were accessible. The Government of Kenya Health centers (Kapsisiywa and Kipsamoite Health Centers) were the only health facilities within the study sites. The topography of the study sites comprises hills, valleys and plateaus. Rivers and streams run along the valley bottoms in the valley ecosystem and 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 highland. The second season is the short rains from the months of October to December, during this 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 to 19°C.

Sampling points for *Anopheles*

Adult *Anopheles* mosquito samples were collected from January to December 2007 in a total of 17 villages within Kipsamoite and Kapsisiywa study sites. Ten percent (n=165) of the households were randomly selected, coded and used as sampling points (Table 1). Larval *Anopheles* sampling was carried out by conducting systematic ground surveys were to determine the possible *Anopheles* breeding sites by sampling aquatic habitats for the occurrence of anopheline larvae. Those habitats positive for anopheles aquatic stages were used as sampling points for *Anopheles* larval forms.

Sampling of outdoor larvae and adult *Anopheles* species

Anopheles larval collection from identified breeding sites was carried out every two weeks from 0600 to 0900 h January to December 2007. A scupper/dipper (13 cm in diameter and 6.5 cm deep) with a handle was used to scope water from the sites. The water was poured onto a white plastic basin/tray for easy collection of larvae by use a plastic pipette. The collected larvae were transferred into small plastic bottles labeled with date, site of collection, and type of habitat. The larval specimens 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 Limited, Gainsville, Fl., USA).

Sampling of indoor adult *Anopheles* species

Total indoor resting mosquitoes were collected from randomly selected households by the pyrethrum space spray method also called pyrethrum spray collection/catches [PSC] method 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 to 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 for preservation on silica gel in Eppendorf tubes prior to species identification.

Identification of *Anopheles* species

All the anopheles mosquito collections were sorted out to separate

Table 1. The number of households used as sampling points.

Study site	Number of villages	Total number of households	Number of households sampled (%)
Kipsamoite	7	666	67 (10)
Kapsisiywa	10	982	98 (10)
Total	17	1648	165

the females from males. The females were identified to species level using morphological features with the aid of identification manual (Gilles and Coetzee, 1987) and *Anopheles* identification soft ware CD. The results are indicated in Table 2. The females of all morphologically identified female *A. gambiae* s.l. mosquitoes collected from houses and those reared to adults from larval mosquitoes were identified to sibling species using PCR assay as described by Scott et al. (1993). The PCR assay involved the following steps: mosquito deoxyribonucleic acid (DNA) extraction using the potassium acetate precipitation technique; making of PCR master mix [mixture of buffer, ions, primers, and enzymes in water]; electrophoresis; gel visualization and photography. Samples not identified after 3 PCR, were marked as unknown. The distribution of bands in the gel after electrophoresis was used to identify and distinguish *A. gambiae* siblings as *A. gambiae sensu strict (s.s)* from *Anopheles arabiensis* species whose oligo primers had been included in the master mix as controls. The results are indicated in Figure 1.

Collection of blood samples and identification of *Plasmodium* species

Ethical approval for the study was obtained from the KEMRI National Ethical Review Committee. Written informed consent for study participation was obtained from all consenting individuals. A blood sample 0.5-1.0 ml was obtained by the following procedure at two health centers: preparation of site (finger) with alcohol, pricking of the fingertip with a lancet and obtaining the blood sample on a clean 25 × 75 mm slide in a 10 to 30 sec period. The blood was spread out to make thick and thin blood smears, dried and fixed in methanol and stained in 4% Giemsa for 30 min. All stained blood films were examined microscopically at 1000× objective under oil immersion to identify the malaria parasite species based on morphological features of sexual and asexual stages. The second and third examination of each smear was done at KEMRI for quality assurance. The whole slide was carefully scanned before being declared negative. Slides were reported negative for parasites only after examining at least 50 fields. The results are indicated in Table 3.

Determination of malaria prevalence sample size

The estimated population of Kipsamoite and Kapsisiywa is 3400 and 300, respectively. The prevalence of malaria was not well known in the study area, therefore a 50 and 25% estimate of the 80% individuals seeking medical care at the health centers, was used for peak and low-transmission seasons, respectively. The sample sizes, from each study site, with a 95% confidence interval and precision level of 5% were arrived at using the formula:

$$n = z^2 (pq) / d^2$$

In this equation, n is the sample size, z is the critical value of the standard normal distribution at the 5% level (1.96), p is the malaria prevalence estimate, q = 1- q, and d is the precision level at 0.05. This translated into approximate sample size of 1325 individuals from Kipsamoite and 615 from Kapsisiywa.

RESULTS

Diversity and abundance of *Anopheles* species

A total of 387 female adult *Anopheles* belonging to 11 species were 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 *Anopheles christyi* New stead and Carter, the most predominant species and ancestor of all malaria vectors (Anthony et al., 1999) followed by *Anopheles demeilloni* Evans, *Anopheles coustani* Levaran, *Anopheles squamosus* Theobald, and *Anopheles harperi* Evans. Other species found in low numbers were: *Anopheles ziemanni* Grunberg, *Anopheles leesoni* Evans and, *Anopheles longipalpis* Theobald. The known human malaria vectors were *A. gambiae, A. funestus,* and *A. arabiensis* comprising 11% in 3 species. *A. 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 indicated in Table 2.

PCR assay for *Anopheles gambiae*

The 41 *A. gambiae* sample specimens collected from the study sites were analyzed by PCR. All were found to belong to one sibling species *A. gambiae s.s* indicating that it was possibly the only sibling species from the *A. gambiae* complex circulation in the study area. For quality assurance and comparative purposes, a run containing both *A. gambiae* and *A. arabiensis* was done to show how the results would have been in case the other sibling species *A. arabiensis* would have been present in the samples. In both cases, single bands were visualized at different levels and photographed for *A. arabiensis* and

Table 2. *Anopheles* diversity, abundance and feeding and vectorial capacities.

Anopheles species	Number of samples and species relative abundance (%)	Known feeding behavior	Vectorial capacity
A. gambiae s.s	41 (10.6)	Anthropophilic	Malaria vector
A . funestus	1 (0.3)	Anthropophilic and zoophilic	Malaria vector, Filariasis vector
A . arabiensis	1 (0.3)	Anthropophilic and Zoophilic	Malaria vector
+*A. christy* (Newstead and Carter)	108 (27.9)	Zoophilic	Not known
A. demeilloni (Evans)	94 (24.3)	Exophilic/zoophilic	Not known
A. coustani (Levaran)	66 (17.1)	Anthropophilic and Zoophilic	Occasional vector of Rift Valley Fever
A. squamosus (Theobold)	51 (13)	Anthropophilic and Zoophilic	Not known
A. harperi (Evans)	21 (5.4)	Anthropophilic and Zoophilic	Not known
A. leesoni (Evans)	2 (0.5)	Zoophilic	Not known
A. longipalpis (Theobold)	1 (0.3)	Anthropophilic	Not known
A. ziemanni (Grunberg)	1 (0.3)	Anthropophili and Zoophilic	Can maintain malaria parasites
Total	387 (100)		

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

Table 3. *Plasmodium* prevalence and parasite species prevalence.

Site	Samples screened	*Plasmodium*			
		P.f (+ve%)	P.m. (+ve%)	Total (+ve)	Prevalence rate
Kipsamoite	1325	86(89.6)	10(10.4)	96	7.2
Kapsisiywa	615	46(90.2)	5(9.8)	51	8.2
Total	1940	132(89.8)	15 (10.2)	147	

P.f +ve% = *Plasmodium falciparum* positive cases expressed as a percentage; P.m +ve = *Plasmodium malariae* positive cases expressed as a percentage; Total +ve = Total positive cases.

A. gambiae s.s. The results are presented in form of photographed gel under ultra violet (UV) light (Figure 1).

Prevalence of malaria parasites

A total of 1940 individuals aged from <1 year and above including males, females were screened for malaria from January to December 2007. The positivity rates (parasite ratio) of infection for the different malaria parasite species is shown in the Table 3. Chi-square analysis indicated a significant difference (χ^2, P=0.03) between *P. falciparum and P. malariae* occurrence in the study sites. *P. falciparum* was the most prevalent malaria parasite accounting for 89.8% of the diagnosed cases in both study sites. *P malariae* was recorded in both sites at low frequency and constituted 10.2% of malaria cases in Kapsisiywa and 9.8% in Kipsamoite. *P malariae* was often seen in mixed infections with *P. falciparum*. No cases of *Plasmodium vivax* and *Plasmodium ovale* were detected during the course of study. Malaria cases were more prevalent between the months of March to August during which about 95% of cases were recorded. The remaining 5% of the cases were detected in January and February. No malaria cases were recorded in October and December 2007 in the study sites.

DISCUSSION

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 human habitations. For the zoophilic and antropophilic species in this group (including *A. christy, A. demillon, A. harperi, A.leesoni,* and *A. longipalpis)*, the initial attraction emanating from cattle/goats and 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

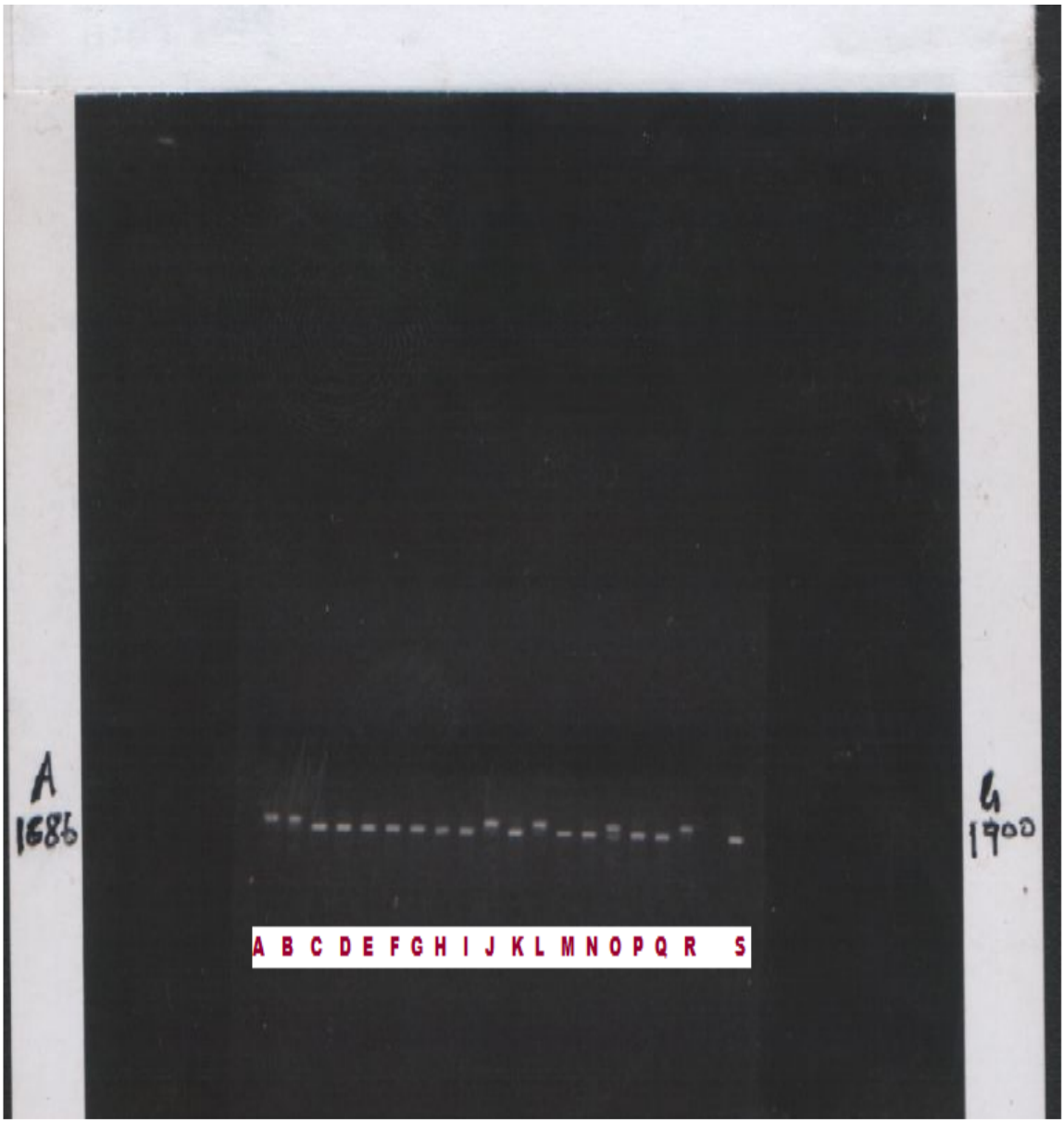

Figure 1. PCR comparative band distribution of *An. gambiae s.s* and *An. arabiensis.* Samples A, B, J,L. O and R were *An. arabiensis* controls; Sample S was *An. gambiae s.s* control; Samples C, D, E, F, G, H, I, K, M, N, P and Q were *An. gambiae* confirmed as *An. gambiae s.s*. Assay conditions: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

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, *A. 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. It is possible that some of the non-vector anopheles species may be of local importance in disease transmission as incidental vectors (Gilles 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 in study area were *A. gambiae, A. funestus*, and *A. arabiensis*. The three *Anopheles* species are known to be the most efficient malaria vectors (Besansky, 1999).

This is because of their marked preference for human environment 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). *A. gambiae* was the most abundant malaria vector and the most prevalent of the three species. These findings were consistence with related studies in the same and neighboring sites as well as other highland areas in Kenya (Petrarca et al., 1991). Previous vector studies in Nandi indicated malaria vector composition of 98% *A. gambiae* complex and 2% *A. funestus* (Kacey et al., 2006). *A. funestus* and *A. arabiensis* were rarely encountered with only one specimen of each collected translating into 2.4% of the total known malaria vectors. This could be because the two species have difficulties to colonize high altitudes successfully. *A. funestus* larvae require up to three weeks to develop into adults compared to only 10 days for *A. gambiae* (Malakooti et al., 1998). 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 western highlands of Kenya. Malaria vector(s) and non-vectors may periodically extend their range beyond their normal area of distribution. This may explain the presence of *A. ziemanni* in study area. The species is widely encountered in West Africa extending to Ethiopia with scanty, localized distribution in East Africa. In Kenya, it has been previously reported mainly in the low lands of Lake Victoria basin (Luna 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, feeds on both man and animals (Luna et al., 2006). The species may be undergoing a phase of adaptation to live in western highlands in proximity to the normal habitat, the lowlands. There is need for further field and experimental studies on *A .ziemanni* as regards possible role in malaria transmission in both low and high altitude areas. The presence of a species of well known and efficient malaria vector, *A .arabiensis*, a predominant vector in lowlands at high altitude raises curiosity. If determined and confirmed in larger long-term studies, the presence of *A .arabiensis* at the present high altitude area would support scanty reports that the species is capable of breeding and transmitting malaria in highland areas (Chen et al., 2006). It is also possible that regular travel between Lake Victoria lowlands and the western highlands could introduce the vector into highlands. Both *A. gambiae s.s* and *A. arabiensis* have similar requirements for their larval environment (Service et al., 1978; Gimnig et al., 2001). Therefore, there is a possibility that *A. arabiensis* imported into the highlands could become established and become an important malaria vector together with *A. gambiae s.s*. Whenever these two species occur together, populations of *A. arabiensis* are known to survive the dry season better while populations of *A. 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.

The relative abundance in Kenya of four *Plasmodium* species that cause human malaria appear as varied as follows: *P. falciparum,* the most common (80 to 85%), followed by *P. malariae* (10 to 15%), *P. ovale* (< 5%), and *P. vivax* reported as infrequent in the human population. In the present study, of the 147 confirmed malaria cases, 89.6 to 90.2% were *P. falciparum* and 9.8 to 10% *P. malariae.* Neither *P. ovale* nor *P. vivax* infections were detected. The relatively short extrinsic incubation periods of *P. falciparum* could be a possible reason why the parasite species is more common than the other *Plasmodium* species (Institute of Medicine, 1991). The species has been associated with frequent malaria outbreaks in the western Kenya highlands in the past (Some, 1994; Githeko and Ndegwa, 2001; Hay et al., 2002a and b). Since *P. falciparum* was the most prevalent in the study area, efforts to limit mortality associated with it must be prioritized because of its potential fatal consequences. It is suggested that any case of malaria encountered in the area should receive effective treatment promptly and early enough to prevent severe disease and death. When *falciparum*- malaria treatment is delayed or not given, there is danger of build-up of chronic infections in the population that eventually spread by the long-lived largely anthropophilic *Anopheles gambiae* vectors leading to outbreaks and epidemics (Marsh and Snow, 1999; Killeen et al., 2000). Shanks et al. (2005) recommended that curing enough confirmed *falciparum*-infections limits seasonal malaria transmission in the western highlands.

Conclusions

In this regard, the choice of the first line malaria therapy is crucial to cure those infected, as well as allow maximum number of persons to get access to treatment. The presence of *A. gambiae s.s,* and *P. falciparum*, the most dominant malaria vector and malaria parasite was established. This suggests that under appropriate conditions the disease can become established and spread rapidly with severe consequences in semi/non-immune local communities. This at policy level calls for regular active disease surveillance and monitoring system and prompt treatment of identified positive cases with appropriate and effective drugs. It would also require the application of current appropriate technology to forestall epidemics from taking root in this highland area. There is also need to educate the communities on the etiology, signs and symptoms of malaria, and prevention and control to enhance their participation in intervention strategies and enhance treatment seeking attitude.

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