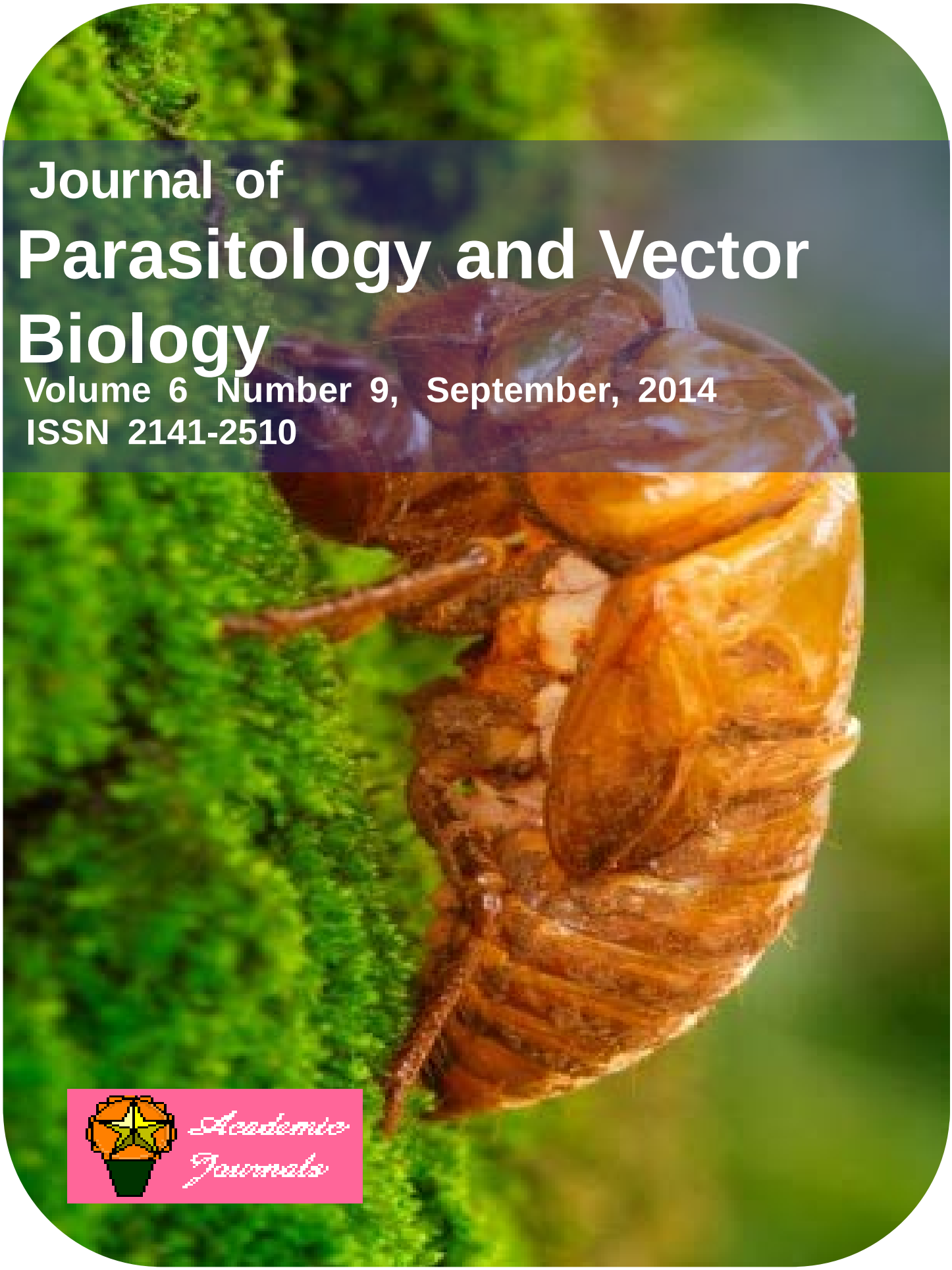

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Full Length Research Paper

Co-infection of HIV and malaria parasites in pregnant women attending major ante-natal health facilities in Akure, Ondo State, Nigeria

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Malaria and human immunodeficiency virus (HIV) in pregnancy are the major factors contributing to adverse maternal and perinatal outcome. HIV increases pregnant women's chances of contracting malaria, increases the risk of developing anaemia, delivering a low birth weight infant and premature delivery. This study was designed to investigate the level of co-infection of malaria parasite and HIV in pregnant women in State Specialist Hospital, Akure. 616 pregnant women aged 15 to 46 years who attended major referral health facilities for ante-natal purposes between February and April, 2012 were included for the study. 'Determine' and Uni-Gold rapid diagnostic tests kits were used to determine HIV status, giemsa stained thick blood smear of patients were examined for presence of the asexual stages of *Plasmodium* parasite. Out of 616 pregnant women, 28 (4.55%) were HIV positive and 597 (96.92%) had malaria parasite. Among HIV negative women, 569 (96.8%) had malaria parasite infection while 28 (100%) of the HIV positive women had the infection. This study revealed that 92.37% had malaria alone, none of the women had HIV only and 28 (4.55%) were co-infected with both pathogens, indicating that all HIV positive women also harbor malaria parasite. There was moderate correlation between HIV and malaria parasite $p < 0.05$ which is suggestive that women infected with HIV are most likely to be infected with malaria due to their compromised immune system which makes them more susceptible to malaria infection. The rate of malaria infection was generally high in the sampled population though majority was infected at low parasiteamia. This is therefore attributed to the endemic nature of the disease in Ondo state of Nigeria

Key words: Human immunodeficiency virus (HIV), malaria, co-infection HIV-MALARIA, antenatal care, pregnant women.

INTRODUCTION

Human immunodeficiency virus (HIV) and malaria are among the leading causes of morbidity and mortality in sub-Saharan Africa, where they represent common infections in women of childbearing age. Together, they

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cause more than four million deaths per year (World Health Organization (WHO), 2011). About 90% of the 300 to 500 million annual acute episodes of malaria are reported in this region, and there is also an estimated 30 million HIV-infected cases (Rowland-Jones and Lohman, 2002). The burden of malaria is particularly severe in children under 5 years (who constitute more than 70% of the 1 million deaths due to malaria) and pregnant women. About 55% of HIV infected adults in sub-Saharan African are women of reproductive age and they account for over 80% of the world's HIV infected women. In this region, the prevalence of maternal malaria is 65% and HIV affects 40% of the pregnant population (Ned et al., 2005). Schantz-Dunn and Nawal (2009) recorded that malaria is one of the most devastating infectious diseases, killing more than one million people annually. Forty percent of the world's population lives in endemic areas. Malaria epidemics have devastated large populations. Consequently the disease poses a serious barrier to economic progress in many developing countries. There are an estimated 300 to 500 million cases of clinical disease per year, with 1.5 to 2.7 million deaths.

Abu-Raddad et al (2006) estimated that, the interaction of malaria and HIV in one Kenyan district alone had caused 980,000 excess malaria episodes and 8,500 excess HIV infections since the emergence of HIV in the 1980s. In areas endemic for malaria, it is estimated that at least 25% of pregnant women are infected with the disease. The highest risk of infection and morbidity is found among the primigravidas, adolescents and those co-infected with HIV (Desai et al., 2007). Malarial infection in HIV-positive women is associated with higher levels of parasitemia, leading to a greater risk of severe anemia. Likewise, HIV viral load is increased, creating opportunity for infection and more severe disease (Brentlinger et al., 2006). As a result of the impaired immune state in pregnant women, HIV infection increases susceptibility to malaria and the morbidity associated with it in co-infected women, resulting in higher incidences of severe anemia and low-birth-weight neonates in co-infected. WHO (2004) explained co-infection of malaria and HIV having disproportionate effects on pregnant women and pose serious risks. Briand et al. (2009) reported that HIV-infected women were approximately 22 times more likely to die than HIV-uninfected women. There was a similar risk for those with malaria. Women with dual infections thus had the greatest risk of death compared with those with only HIV infection or malaria infection. Malaria infection in HIV-positive pregnant women can also increase the risks of mother to child transmission (MTCT) during pregnancy, labor as well as during breastfeeding period due to increase in the level of HIV in the blood, that is increase in viral load (UNICEF, 2003). Thus any interaction between malaria and HIV can be an additional health burden, especially in pregnant women, with resulting poor birth outcomes and the imminent transmission of these intracellular pathogens. Adeoti et al. (2012) studied prevalence of HIV and malaria parasites co-infection in pregnant mothers and their babies post-delivery, clearly showing that the recruitment profile of the samples examined exhibited prevalence of malaria infections in the HIV infected mothers and their infants. The aim of this research work is to determine the prevalence and the level of co-infection of HIV and malaria infection in pregnant women at Akure, Ondo State, Nigeria using State Specialist Hospital Akure.

MATERIALS AND METHODS

An ethical clearance was sought and collected from the Ondo State Ministry of Health for permission to carry out the present research that involved human subject. The study subjects consisted of 616 pregnant women aged 15 to 46 years, who came from different local government areas, for antenatal purposes at Ondo State Specialist Hospital located in Akure, between February and April, 2012. They were selected randomly without the prior knowledge of their clinical and family history.

Sample collection

Venous blood was collected from the upper arm of the pregnant women with the assistance of trained Laboratory Scientists, following the method described by cheesebrough (2005). 5 ml of blood needed for the tests was drawn gently at site selected, using sterile needle and syringe into the anticoagulant (EDTA) bottles. Each sample bottle was labeled to correspond to the age and number of subject at the point of collection.

Determination of human immunodeficiency virus (HIV) status of pregnant women

Standard commercially sourced 'Determine' and "Uni-Gold rapid diagnostic tests kits were used to determine the HIV status of the subjects. 'Determine' HIV Rapid Test kit (For use with whole blood, serum, or plasma) (www.who.int/diagnostics laboratory). Uni-Gold test kits are kits that pick or react to only HIV in the blood sample (The Trinity Biotech *Uni-Gold*™ HIV test).

"Determine diagnostic test kit" procedure

The expiration date of kit was checked to ensure the kit had not expired, after which the protective wrapper was removed from the test device and the kit labelled with the patient identification number. Disposable pipette was filled with blood sample and held over the sample pad marked by an arrow symbol into which two drops of sample was carefully added. Blood was allowed to absorb into the sample pad and result was read and recorded after waiting for 60 min. Any positive result was subjected to Uni-Gold test to confirm the positivity of the test result.

Table 1. Age distribution of HIV antibodies among pregnant women.

Age group (years)	No. examined	No. positive	Percentage positive
15-19	13	1	7.69
20-24	49	2	4.08
25-29	211	9	4.27
30-34	222	11	4.96
35-39	104	5	4.81
40-44	14	0	0
≥45	3	0	0
Total	616	28	4.55

Uni-Gold diagnostic test kit procedure

The expiration date of kits was checked to ensure they had not expired, after which the protective wrapper was removed from the test device and the kit was labelled with the patient identification number. Disposable pipette was filled with sample and held over the sample pot, into which two drops of sample was carefully added. Two drops of appropriate wash agent was also added to the sample pot. Result was read and recorded after waiting for 20 min for reaction to occur. The appearance of a line in the control region indicates a negative test result while a line in both the test and control regions is indicative of a positive result. When no line appears in the control region, the test was regarded as inconclusive and consequently repeated with a fresh kit.

Malaria diagnosis in pregnant women

All thick films were prepared and stained with Giemsa stain using a modification of method described by Cheesebrough (2005). The approximate numbers of parasites were reported using plus sign: 1 to 10 parasites per 100 high power fields as +, 11 to 100 parasites per 100 high power fields as ++, 1 to 10 parasites in every high power field as +++, and if no parasites were found after examined as NPF (Cheesbrough, 2005). This was done by a trained and experienced laboratory scientist.

Analysis of data

Analysis of data was carried out using MS-Excel and SPSS version 16.0 (SPSS Chicago Inc., IL, and U.S.A). Pearson correlation analysis was used to investigate possible relationship or correlation between HIV and malaria positivity. Statistical significance was set at p value < 0.05.

RESULTS

Prevalence was calculated by comparing the number of positive individuals with the total number examined. Also, for the analysis, the population was grouped into seven age categories and for each age group of pregnant mothers, the number positive and infection rate per category was estimated.

Results of HIV screening in different age groups of pregnant women

Result in Table 1 showed that out of the six hundred and sixteen sampled populations, 28 women representing 4.55% were positive to HIV infection. The prevalence of HIV antibodies occurred most among women aged 15 to 19 (7.69%) followed by those aged 30 to 34 years (4.96%) while none of the women aged 40 years and above was found to be infected by the virus. The distribution of infection among other age groups of women did not follow any particular pattern. For instance, women aged 20 to 24 had (4.08%), 25 to 29 had (4.27%), 30 to 34 had (4.96%), and 35 to 39 years had (4.81%). Result also shows that HIV infection rate decrease as age of women increases, with the exception of mothers aged 25 to 29 and 30 to 34 years, where infection rate increased slightly from 4.27 to 4.96%.

Prevalence of malaria parasitaemia in the different age groups of pregnant women

In Table 2, a total of 597 pregnant women representing 96.92% of those tested had malaria infection. The infection was generally high in the sampled population. Women aged 15 to 19 and 40 years and above were all infected with malaria parasite. The rate of infections increased steadily through the age group, starting from age 20 to 24, with 95.92%, up to age 35 to 39 years, with 99.04%.

Characterization of malaria parasite density in relation to age of positive pregnant women

The density of malaria parasite in the different age groups of the 597 infected pregnant women was categorized as follows: (+) low, (++) moderate and (+++) high parasiteamia. Result indicated that malaria with very high

Table 2. The prevalence of malaria parasite among the different age groups of pregnant women.

Age group (years)	No. examined	No. positive	Percentage positive
15-19	13	13	100
20-24	49	47	95.92
25-29	211	203	96.21
30-34	222	214	96.39
35-39	104	103	99.04
40-44	14	14	100
≥45	3	3	100
Total	616	597	96.92

Table 3. Characterization of malaria parasite density in relation to age of positive pregnant women.

Age group (years)	Malaria parasite density		
	Low (+)	Moderate (++)	High (+++)
15-19	12	1	0
20-24	42	5	0
25-29	187	16	0
30-34	201	12	1
35-39	97	6	0
40-44	14	0	0
≥45	3	0	0
Total	556 (93.17%)	40 (6.7%)	1 (0.17%)

Age long medically accepted mode of classification of malaria parasite density in patients.

high parasiteamia was found only in pregnant women aged 30 to 34 (0.17%), moderate parasiteamia was found in mothers aged 15 to 39 (6.7%) while the older ages were not infected, and low parasiteamia was found in all the age groups (93.17%) (Table 3).

Result of co-infection of HIV and malaria parasites among pregnant women

Result obtained on the co-infections of HIV and malaria parasites in this present study ascertain the proportion of pregnant women carrying concurrent infections of both HIV and malaria. This study revealed 28 (4.55%) positivity to both pathogens, 92.37% had malaria alone and none of the women had HIV only (Table 4). Pearson's correlation coefficient (r) of 0.5 shows moderate correlation. There was a significant correlation ($P < 0.05$) between HIV and malaria parasite infection in the pregnant women (Table 5).

DISCUSSION

The present 4.55% prevalence of HIV antibodies in Akure, Ondo State was not surprising based on the number of AIDS cases reported from Nigeria (WHO, 1995). In addition, personal observation showed that practices identified as high risk factors in the contraction and transmissions of HIV infections (Olusi et al., 1996; Olusi, 1998) are common in the state. Such practices include prostitution, keeping of multiple sex partners, use of unsterilized or partially sterilized needle and syringes by qualified and unqualified health workers, especially in mission houses. As a result of these, the majority of persons in the study group can be assumed to have a history of risky practices.

The differences in the pattern of HIV infection in the age group of pregnant mothers in this study has to do with the known epidemiological features of HIV infection (Olusi et al., 1996). The absence of HIV antibodies among women aged 40 years and above suggested that

Table 4. Prevalence of HIV and Malaria parasite Co-infections among pregnant women in Akure.

Infection	No. examined	No. positive	Percentage positive
HIV and Malaria	616	28	4.55
Malaria alone	616	569	92.37
HIV alone	616	0	0

Table 5. Correlation of malaria parasite and HIV.

Parameter		Value	Asymptotic standard error (a)	Approximation T (b)	Approxinmate significance
Interval by Interval	Pearson's R	0.500	0.074	14.078	0.000 (c)
No. of valid cases		597	-	-	-

[a]Not assuming the null hypothesis. [b]Using the asymptotic standard error assuming the null hypothesis. [c]Based on normal approximation.

most of the women in this age group had formal education about the infection and took extra care by keeping to one sex partner and more so that they are in their late reproductive ages, while other age groups could probably have contracted the HIV antibodies through heterosexual intercourse and carefree attitude, most especially among the young pregnant women aged 15 to 19 years that are still sexually active; these therefore probably indicates the reason for the prevalence and distribution of HIV infection among pregnant women in Akure, Ondo State.

The present work is in contrast to the research work done at Cameroon by Nkuo-Akenji et al. (2011) who observed the prevalence rate of 21.1% HIV infection and a higher number (60.7%) of HIVSP patient in ages 26 to 35 and 14.1, 20.0 and 0% in ages 15 to 25, 36 to 45 and > 45, respectively. Perrault (2009) reported mean HIV prevalence in pregnant women attending antenatal clinics in South Africa, where he noted prevalence rate of 15 to 40% in South Africa but a lesser prevalence of 5 to 10% in East Africa. Adeoti et al. (2012) also exhibited 19 (12.8%) prevalence of HIV infection among 149 pregnant mothers examined.

The result obtained in the present study indicates that 96.92% of the population of pregnant women examined had malaria parasite, though majority (93.17%) of the mothers were infected at low parasitaemia, which is suggestive of the reason why they did not come down with malaria despite being infected. This indicated that pregnant women could be carriers of malaria parasites without showing any symptoms to the infection. The high infection rate in this study therefore is attributed to the endemic nature of the disease in Ondo state of Nigeria.

The results obtained in this work and those of previous studies support the endemic nature as well as in other part of the country. For instance, Martra et al. (1993) found *Plasmodium falciparum* affecting 97.27% cases of pregnant women examined. Falade et al. (2008) found 89% asymptomatic malaria parasite among pregnant women attending antenatal in a Secondary health care facility in Ibadan, Nigeria. Adefioye et al. (2007) also recorded 72% prevalence rate and Chukwurah et al. (2003) recoded 63.5% of prevalence of malaria parasite in pregnant women in Akwa Ibom, Nigeria. The high infection rate observed among women aged 15 to 19 and 40 years and above is probably an indication of low immunity to malaria infection due to primigravidae status of pregnant women (first pregnancy), and so they are more susceptible to malaria infection than others (Martra et al., 1993). Immunity to malaria infection diminishes significantly in pregnancy, particularly in primigravidae (Oforie et al., 2009). In first and second pregnancies, women are especially vulnerable to malaria infection. Mcgregor (1984) identified the factors responsible for susceptibility of primigravidae to malaria as inhibition of type 1 cytokine responses (interferon, interleukin 2 and 12 and TNF). Brabin (1991) also confirmed that the primigravidae were more susceptible to malaria infection than the multigravidae. Opare Addo et al. (2002) reported that primigravidae, alongside those women in their second pregnancy, were more vulnerable to malaria parasitemia. This work is in contrast to the work of McGregor (1983) who reported a decline in malaria prevalence as age increase, as well as with improved host immunity, thus reducing susceptibility in later years.

The percentage of women carrying both infections in the study indicated that all the pregnant women that were positive to HIV antibodies also habour malaria parasites. This research work compared with other recent studies, Adeoti et al. (2012), observed that the prevalence of

malaria infections in HIV infected mothers was due to the impaired immune status of HIV infected pregnant women to control *P. falciparum*. It was also observed that the pregnant women that are infected with the HIV severely suffered from malaria. Previous studies indicate that people with haemoglobin genotype AS are not prone to malaria susceptibility when they have high level of immunity, but from this study high parasitaemia (+++) was observed from a pregnant woman with genotype AS. The findings in this study further support previous findings that dual infection with HIV and malaria may have serious consequences on the health of pregnant women. The dual infected pregnant women could be susceptible to many other infections. This is the evidence that where malaria and HIV occur together, infections interact. HIV worsens malaria infection by lowering the immunity of the infected pregnant women while malaria increases the risk of transmitting HIV from mother to child and also HIV viral load in the infected pregnant women is increased.

Desia et al. (2007) reported that in the areas endemic for malaria, at least 25% of pregnant women are infected with malaria, with the highest risk for infection and morbidity in primigravidas, adolescents, and those co-infected with HIV. Ned et al. (2005) also observed that the HIV co-infection has its impact on disease presentation, with an increased risk of complicated and severe malaria and death. Malarial infection in HIV-positive women is associated with higher levels of parasitemia, leading to a greater risk of severe anemia. Likewise, HIV viral load is increased, creating opportunity for infection and more severe disease (Briand et al., 2009). The moderate correlation that occurs between malaria and HIV implies that mothers infected with HIV are most likely to be infected with malaria due to the compromised immune system which makes them more susceptible to malaria infection.

RECOMMENDATION

It is therefore recommended that all pregnant women attending antenatal clinic in State Specialist Hospital Akure be tested for malaria parasite every month, in order to detect early, in case of any symptomatic or asymptomatic malaria infection, so as to be treated appropriately and prevent further morbidity and mortality associated with malaria. Intermittent preventive treatment should be provided for all pregnant women with preventive doses of an effective anti-malarial drug during routine antenatal clinic visits. Mosquito treated net should be made available free for the pregnant women. Training on how to use and maintain the net must be given as this will decrease the exposure to infective mosquito bites. More impressive public announcement and awareness should be made to the pregnant women in the public to attend good antenatal clinic for proper handling of their health and pregnancy from malaria and HIV.

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Conflict Interests

The authors declare that there is no conflict of interests

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Full Length Research Paper

Determination of insecticidal effect (LCD_{50} and LCD_{90}) of organic fatty acids mixture (C8910+silicone) against malaria vectors

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Malaria vectors have acquired widespread resistance to several insecticides; thus, there is a critical need for the development of alternative insecticides for use in vector control programs. The mosquito toxicity of a novel insecticide/repellent consisting of medium-chain carbon fatty acids (C8910) was examined. Determination of LCD_{50} and LCD_{90} was made against six colony-reared *Anopheles* species using probit analysis on mortality data generated by Centers for Disease Control and Prevention bottle bioassays. Eight different concentrations of C8910+silicone oil provided an LCD_{50} ranging from 55.4 (44.2 to 65.9) in *Anopheles minimus* to 132.6 (92.8 to 301.3) in *Anopheles dirus*. Similarly, LCD_{90} varied from 138.5 (107.9 to 207.9) to 1228.8 (449.8 to 21400), respectively. Further development of C8910 and similar compounds could provide vector control specialists novel, environmentally-safe insecticides for controlling insect disease vectors.

Key words: LCD_{50}, LCD_{90}, bottle bioassay, C8910, silicone, malaria, *Anopheles*.

INTRODUCTION

There is an increasing need for alternative insecticides and repellents to control disease-transmitting arthropods. The lack of available, novel insecticide classes for vector control has left experts with a limited set of chemicals to manage insecticide resistance. The emergence of vector resistance to insecticides currently deployed in malaria control programs, for example, can severely impact the effectiveness of principle interventions such as indoor residual spraying (IRS) and long-lasting insecticide-treated bed nets (Ochomo et al., 2013). Since the introduction of synthetic pyrethroids more than 30 years ago, no new mosquito adulticide classes have been

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approved for vector control by the World Health Organization (WHO) (Nauen, 2007). Because of this, compounds unrelated to the four classes (that is, organochlorines, organophosphates, carbamates, and pyrethroids) need to be investigated.

Research using plant essential oils and animal by-products as alternative compounds for arthropod control and repellency is growing (Isman, 2006; Chansang and Mulla, 2008; Hieu et al., 2010; Mng'ong'o, 2011). Plant oils and fatty acids are common in nature and have been shown to provide insecticidal or repellency effects against a variety of arthropods (Dolan et al., 2007; Chansang, 2008; Mullens et al., 2009; Hieu et al., 2010; Cantrell et al., 2011; Ali et al., 2012). C8910, a mixture of medium-chain octanoic (CH3(CH2)6COOH), nonanoic (CH3(CH2)7COOH) and decanoic (CH3(CH2)8COOH) acids in equal parts, discovered and developed by Stratacor, Inc., (Richmond, CA), has shown promise as both an insecticide and repellent (at higher doses) when formulated appropriately with carriers such as silicone oil or kaolin clay (Reifenrath, 2001). The average molecular masses of the three fatty acids (C8 = 144.2; C9 = 158.2; C10 = 172.3 g/mol) are less than half of dichloro-diphenyltrichloroethane (DDT) (=354 g/mol); thus, provide more than twice the number of fatty acid molecules compared to the same weight of DDT for example. Octanoic and decanoic acids are derived from palm kernel oil or coconut oil and nonanoic acid is derived from tallow; all of these products are inexpensive commodity chemicals. These fatty acids have been approved by the United States Food and Drug Administration (US FDA) as food additives in the U.S. since 1965 and are categorized as "Generally Recognized as Safe" (Reifenrath, 2001). The combination of these acids has shown to have selective repellent and/or toxic effect against mosquitoes and a variety of flies (Mullens et al., 2009; Reifenrath, 2006). Whereas, spiders, bumble bees, honey bees, and wasps are relatively insensitive (Reifenrath, 2001).

C8910 has shown repellency against house flies, biting flies, ticks, ants, sand flies, and mosquitoes [*Aedes aegypti* (L.)] (Reifenrath, 2006); however, no formal studies have been conducted to determine effective concentrations and insecticidal effects of C8910 against malaria vectors. In order to begin to determine an effective concentration of C8910 comparable to WHO Pesticide Evaluation Scheme (WHOPES) approved insecticides, the Centers for Disease Control and Prevention (CDC) bottle bioassay procedure was used to determine the LCD_{50} and LCD_{90} of this novel insecticide/repellent against six colony-reared, susceptible (to all insecticide classes) *Anopheles* species (*Anopheles dirus* Peyton and Harrison, *Anopheles farauti* Laveran, *Anopheles freeborni* Aitken, *Anopheles gambiae* Giles, *Anopheles minimus* Theobald, and *Anopheles stephensi*) representing species known from different geographical regions (Southeast Asia, South Pacific, North America, Africa, Southeast Asia/India, and India/Middle East, respectively). The primary objectives of this study were to determine the insecticidal effects, if any, against male and female *Anopheles* species and to establish baseline effective concentration data that can be used to further develop C8910 for controlling malaria vectors.

MATERIALS AND METHODS

Bottle bioassays

Insecticide susceptibility tests were performed at CDC Atlanta, GA using the CDC bottle bioassay under normal room conditions (23° C; RH ~50%) following protocols established by Brogdon and Chan (2010). Bottle bioassays were used to evaluate a range of concentrations to determine an effective concentration of C8910. Dose-fixing concentrations ranging from 15 to 120 µg C8910/bottle (250 ml glass Wheaton bottle; inside surface area = 370 cm^2) were used to compile mortality data. An initial stock solution of 10 mg (= 10.7 µl) of 15% C8910 + 85% silicone oil (silicone oil is a benign carrier and used with acetone allowed the C8910 to adequately treat and dry on glass bottles) in 100 ml acetone (carrier and drying agent) was made and provided a concentration (= dose) of 15 µg C8910 + silicone/bottle. Additional concentrations of 30, 45, 60, 75, 90, 105, and 120 µg C8910 + silicone/bottle were sequentially made after bioassays using lower concentrations of C8910 indicated mortality was not achieved. Brogdon and Chan (2010) recommend that it is best to align the dose so that 100% of mosquitoes are dead by 1 h or less. For the purposes of this study, we used a relatively low concentration of C8910 (15% C8910/85% silicone oil) tested previously (Reifenrath, 2001, 2006; Mullen et al., 2009) and conducted mortality counts up to 2 h. In order to provide comparative results to other WHOPES-approved insecticides, mortality data for the 30 min time interval (Brogdon and Chan, 2010) were used for LCD_{50}/LCD_{90} determination. Dose-fixing followed the procedures recommended by Robertson et al. (2007). Specifically, it is recommended that for estimation of LCD_{50} and LCD_{90}, a minimum of 4 to 5 doses using at least 200 test insects per dose are required. All C8910 + silicone/acetone stock solutions were stored at room temperature in a dark cabinet.

1 ml of stock solution for each concentration was deposited in each bottle to coat the inside surface and was allowed to air dry for approximately 24 h. One ml of acetone was used to treat the inside of the control bottle. Four treated bottles and one control bottle were used for each assay and each assay was considered one replicate. A total of five replicates (= bioassay rounds) for each concentration were performed. Replicates were not all performed on the same day. When coating/treating the inside surface of the bottles, bottles were slowly turned and rolled with the cap on to evenly treat the inside surface of the bottle and cap, and were checked a few minutes later and rolled again to make sure no 'puddling' occurred on the bottom of the bottle (doing this allowed for more thorough drying). Bottles were placed on their sides with caps off and left to dry overnight. Drying the bottles overnight ensured that the C8910/acetone mixture was dry before introducing mosquitoes and may have resulted in loss of volatiles, thus providing a conservative protocol.

Two to five day old, colony-reared *Anopheles* species (susceptible to all insecticide classes) were introduced to bottles and observed for up to 2 h with mortality counts made at 15 min intervals.

Table 1. LCD_{50} and LCD_{90} (C8910+silicone oil /bottle) of *Anopheles* sp. tested after 30 min exposure.

Species	LCD_{50}	Upper / Lower	LCD_{90}	Upper / Lower	Slope±SEM
An. dirus	132.592	301.263 92.889	1228.8	214.00 449.80	1.325±0.088
An. farauti	118.170	192.867 100.074	243.855	1249.959 164.114	4.073±0.273
An. freeborni	119.867	162.566 98.495	628.577	1599.870 372.223	1.781±0.101
An. gambiae	91.762	110.020 79.852	242.266	424.510 178.755	3.040±0.130
An. minimus	55.444	65.916 45.167	138.475	216.910 107.891	3.224±0.110
An. stephensi	112.576	212.715 86.986	329.757	2472.405 186.821	2.746±0.131

The upper and lower 95% confidence limits are also shown (Statistical analysis by PoloPlus 2.0). *An* = *Anopheles.*

The six species tested herein have been in colony at their current location (CDC, Roybal Campus, Atlanta, GA) since 2010; overall age of colonies and susceptibility testing history was not determined. Mosquitoes were randomly aspirated out of rearing containers and gently aspirated into bottles, and no attempt was made to separate (or count) males from females. Because mosquitoes were two to five days old, a reasonable assumption can be made that approximately 50% of each sex was represented in each test bottle and on average for all bioassay rounds. Approximately 15 to 25 mosquitoes were introduced to each bottle and the timer was started once all five bottles had a mosquito population. For counting purposes during assays, a mosquito was considered dead if it was unable to stand (Brogdon and Chan, 2010). During some of these assays, a few mosquitoes at lower concentrations would recover to a certain degree only to eventually die. These individuals were counted as alive at said time interval if they appeared to revive. To ensure a mosquito counted as dead at earlier time intervals did not recover fully, assays were conducted up to two hours. There was no category for moribund individuals. The bottle was tapped a few times before a final determination of dead or alive was made.

Determination of LCD_{50} and LCD_{90}

Percent mortalities, obtained for each concentration at 30 min, were plotted in a log-probit graphic using Polo-plus 2.0 (LeOra Software Company®, Petaluma, California; 2005). Percent mortalities and LCD_{50}/LCD_{90} analyses were calculated by combining the total individuals and number of individuals (assuming each sex was represented equally) that responded (= died) during each assay (that is, total individuals and total that responded in each bottle were analyzed using the aggregate of the four treated bottles per assay). Control totals were entered into Polo-plus as the aggregate of the five replicates for all eight concentrations. Parameters for data files analyzed by Polo-plus were as follows: probit model, natural response, and concentrations converted to logarithms. The LCD_{50} and LCD_{90} were then obtained together with 95% confidence upper and lower limits.

RESULTS

LCD_{50} and LCD_{90} and percent mortality

The C8910 + silicone formulation evaluated herein did not provide significant mortality at the concentrations tested; however, percent mortalities do increase over time and at higher concentrations. Eight different concentrations of C8910 + silicone oil provided an LCD_{50} ranging from 55.4 (44.2 to 65.9) in *An. minimus* to 132.6 (92.8 to 301.3) in *An. dirus*. Similarly, LCD_{90} varied from 138.5 (107.9 to 207.9) to 1228.8 (449.8 to 21400), respectively (Table 1). C8910 worked best against *An. minimus* and *An. gambiae*; both showed lower values of $LCD_{50/90}$ accompanied by narrow 95% CI when compared to $LCD_{50/90}$ estimates for the other four vector species tested. *Anopheles dirus*, *An. farauti*, *An. stephensi* and *An. freeborni* showed higher values of $LCD_{50/90}$. Mortality percentages at 30 min after being exposed to the higher C8910 doses was close to 50% in *An. dirus*, *An. freeborni*, *An. gambiae*, and *An. stephensi*. PoloPlus

Table 2. Average percent mortality of *An. dirus* (up to 120 min) in bottles treated with C8910+silicone oil.

Concentration (µg/bottle)	15	30	45	60	75	90	105	120
15 min	3.8	5.8	10.4	7.6	22.0	26.0	22.0	34.8
30 min	17.0	13.6	24.2	31.6	43.2	44.6	38.6	54.0
45 min	31.0	22.2	33.0	48.0	57.2	60.4	51.6	70.3
60 min	37.2	31.6	44.8	56.8	67.8	74.0	65.0	79.3
75 min	46.6	42.8	58.6	64.0	76.8	80.2	73.4	85.9
90 min	49.6	48.0	66.8	70.8	85.0	86.2	81.4	88.5
105 min	60.0	56.4	73.4	74.4	86.8	90.0	88.6	91.2
120 min	63.4	63.8	76.0	79.6	89.8	92.8	90.4	92.8
Control (at 120 min)	1.0	1.7	1.9	0	0	0	1.0	0.0
N	5	5	5	5	5	5	5	5

N =Total number of assays (1 assay=4 treated bottles and 1 control bottle).

Table 3. Average percent mortality of *An. farauti* (up to 120 min) in bottles treated with C8910+silicone oil.

Concentration (µg/bottle)	15	30	45	60	75	90	105	120
15 min	1.0	0.8	3.2	3.4	11.6	16.6	19.8	45.2
30 min	1.8	3.0	8.4	11.8	22.2	27.4	32.6	68.2
45 min	4.6	8.2	13.0	17.8	20.4	44.4	44.6	81.1
60 min	7.0	12.8	18.6	23.0	36.0	52.2	57.0	86.1
75 min	9.8	15.6	22.4	31.4	44.4	64.0	70.4	88.5
90 min	11.6	20.0	25.6	36.4	51.4	72.4	80.0	92.0
105 min	16.0	24.4	31.0	43.8	60.6	80.0	88.4	93.4
120 min	19.6	31.0	35.4	51.8	67.2	86.4	92.4	96.9
Control (at 120 min)	1	2.7	2.8	2.2	0.9	0	3.4	4.2
N	5	5	5	5	5	5	5	5

N=Total number of assays (1 assay=4 treated bottles and 1 control bottle).

regression lines showing percent response at 30 min for each species are included in Figures 1 to 6. Tables 2 to 7 show percent mortalities for each concentration at each time interval up to 120 min.

DISCUSSION

We evaluated a relatively low concentration C8910 formulation here to mirror previous studies. Mortality percentages (Tables 2 to 7) show that the relatively low concentration of C8910 used for these bioassays took longer than 30 min to provide greater than 90% mortality (for most species this was not achieved until 105 min or longer); however, PoloPlus software was able to predict LCD_{90} for test subjects (Table 1), showing that higher concentrations of C8910 + silicone is required to achieve LCD_{90}. This study provides and initial attempt to determine the insecticidal effects of one C8910 formulation; higher concentrations should be evaluated to demonstrate stronger C8910 efficacy. The senior author of this work has noted that pure C8910 (33% of each carbon) will incapacitate colony-reared *An. gambiae* within 5 min and kill all mosquitoes in test bottles by 30 min at concentrations of 500 µg C8910/bottle (unpublished data). Further tests against wild *Anopheles* populations are required, as colony strains may have reduced vigor. Concentrations of C8910 required for sufficient control of wild populations may be higher than those observed or predicted by PoloPlus herein. While we tested here both male and female mosquitoes to determine if C8910 + silicone demonstrated any sex-linked susceptibility differences, it is recommended that future studies use females only or sexes are tested separately; however, we did not note any differences in overall mortality between male and females during testing. Aïzoun et al. (2014) conducted CDC bottle bioassays using both sexes and noted that even though male *An. gambiae* were smaller

Table 4. Average percent mortality of *An. freeborni* (up to 120 min) in bottles treated with C8910+silicone oil.

Concentration (µg/bottle)	15	30	45	60	75	90	105	120
15 min	0.9	7.4	9.6	16.4	21.1	23.8	40.6	34.8
30 min	5.6	15.2	23.5	29.5	35.1	33.1	56.4	52.9
45 min	9.2	23.0	32.2	39.2	41.2	40.5	68.6	74.5
60 min	13.7	29.6	37.7	43.5	49.9	51.5	74.9	75.8
75 min	16.2	38.4	42.2	47.3	57.1	57.4	80.3	81.3
90 min	19.9	42.3	48.0	53.8	63.1	64.7	81.7	84.8
105 min	25.0	47.0	52.3	57.4	68.7	68.6	83.5	85.7
120 min	27.5	49.5	53.9	60.2	71.5	71.6	85.4	89.0
Control (at 120 min)	0.87	5.8	6.1	0	2.8	1	1	4
N	5	5	5	5	5	5	5	5

N=Total number of assays (1 assay=4 treated bottles and 1 control bottle)

Table 5. Average percent mortality of *An. gambiae* (up to 120 min) in bottles treated with C8910+silicone oil.

Concentration (µg/bottle)	15	30	45	60	75	90	105	120
15 min	0.0	0.6	7.9	23.7	19.3	33.7	41.5	42.0
30 min	0.0	3.1	23.8	35.3	38.2	54.5	55.8	58.0
45 min	1.1	6.3	32.6	45.0	46.7	67.7	67.9	65.0
60 min	3.6	11.8	40.3	52.2	53.2	73.7	71.8	72.8
75 min	5.2	14.9	44.0	56.0	55.9	78.1	76.0	80.6
90 min	7.5	18.6	46.2	59.8	58.7	80.6	77.9	83.5
105 min	8.8	21.0	47.9	60.8	60.1	81.6	82.4	89.3
120 min	45.6	23.9	51.9	62.5	63.1	84.4	85.3	92.9
Control (at 120 min)	3.6	8.6	2.8	1	2.1	5.0	2.8	12.1
N	5	5	5	5	5	5	5	5

N=Total number of assays (1 assay=4 treated bottles and 1 control bottle).

Table 6. Average percent mortality of *An. minimus* (up to 120 min) in bottles treated with C8910+silicone oil.

Concentration (µg/bottle)	15	30	45	60	75	90	105	120
15 min	0.0	2.2	19.7	38.6	50.0	49.0	58.1	69.4
30 min	1.2	13.5	47.6	63.6	72.1	68.4	72.7	89.1
45 min	5.9	25.5	58.5	72.5	83.4	75.5	78.8	94.1
60 min	8.3	32.9	62.7	80.2	86.7	82.0	84.9	96.2
75 min	9.6	40.5	64.4	82.3	92.4	86.5	89.7	98.2
90 min	10.0	46.7	69.6	83.7	94.6	91.5	91.2	98.4
105 min	13.6	50.3	74.9	83.9	96.3	93.4	92.6	98.6
120 min	15.3	53.8	79.8	86.0	97.8	95.6	92.8	99.3
Control (at 120 min)	0.0	0.0	3.0	0	2.0	0.0	0.9	0.0
N	5	5	5	5	5	5	5	5

N=Total number of assays (1 assay=4 treated bottles and 1 control bottle).

smaller and more fragile in appearance, there was no difference in susceptibility to permethrin. C8910's effect is fairly rapid on both sexes (within minutes of initial exposure, especially at higher concentrations), and the first signs of effect include rapid wing beats with individuals making only brief flights or hovering close to the bottom of the bottle. Time to death (that is, individuals without any movement) may take several hours, especially when

Table 7. Average percent mortality of *An. stephensi* (up to 120 min) in bottles treated with C8910+silicone oil.

Concentration (µg/bottle)	15	30	45	60	75	90	105	120
15 min	0.4	0.7	0.9	13.8	18.1	16.8	31.7	29.2
30 min	0.9	3.6	7.9	29.3	49.7	37.0	56.8	53.6
45 min	1.5	7.5	18.6	52.8	59.8	52.1	74.8	66.8
60 min	3.4	13.4	25.2	61.7	74.2	65.9	84.4	78.2
75 min	10.3	21.4	34.3	68.4	82.4	74.0	87.5	84.5
90 min	14.3	26.9	44.7	75.1	86.7	79.8	90.8	88.7
105 min	16.7	32.0	48.8	80.1	89.3	83.3	94.4	91.4
120 min	17.9	40.1	52.4	83.0	90.7	87.3	94.6	94.2
Control (at 120 min)	2.0	0.8	2.7	0.8	0	0	2.8	0.8
N	5	5	5	5	5	5	5	5

N=Total number of assays (1 assay=4 treated bottles and 1 control bottle).

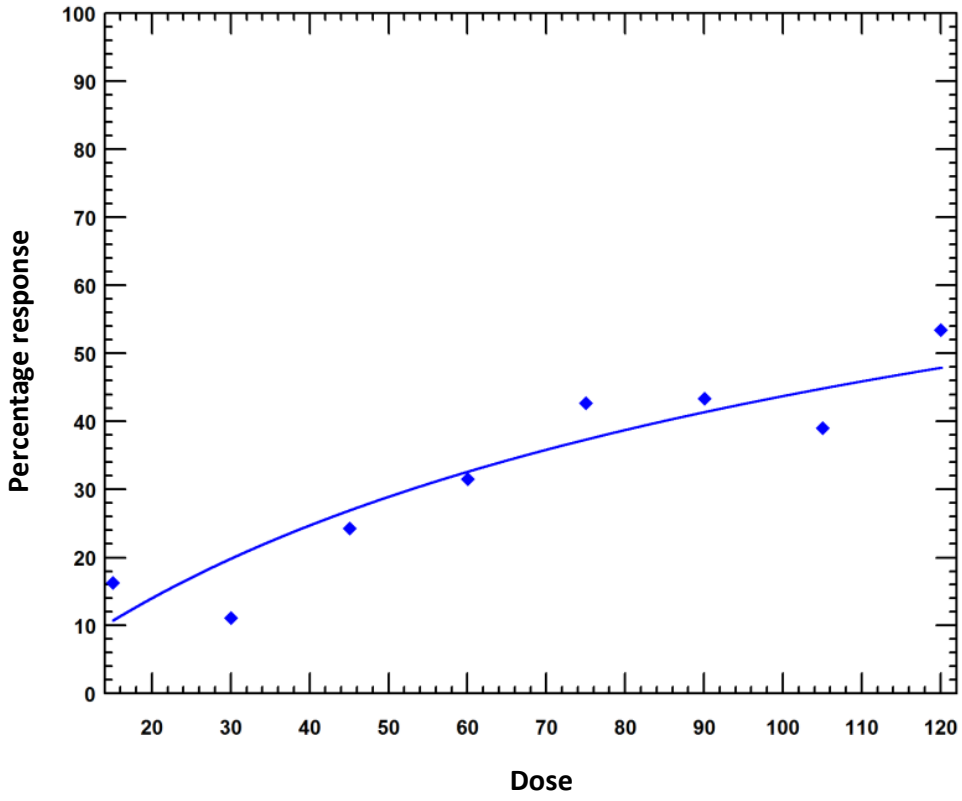

Figure 1. PoloPlus regression line showing percent response (= mortality) of *An. dirus* to C8910+silicone oil at 30 min.

exposed to the lower concentrations tested here. Several individuals were routinely observed attempting to fly after appearing 'dead' when the bottle was tapped/disturbed. Mosquitoes tested against the concentrations used during this study appeared to have an extended moribund period between knockdown and death but do eventually die as no individuals in treated bottles were alive 24 h post exposure. At higher concentrations of C8910, individuals were incapacitated at within 5 to 10 min following exposure.

C8910 in its basic form is an oily substance with a slight coconut odor, similar to sun tan lotion products.

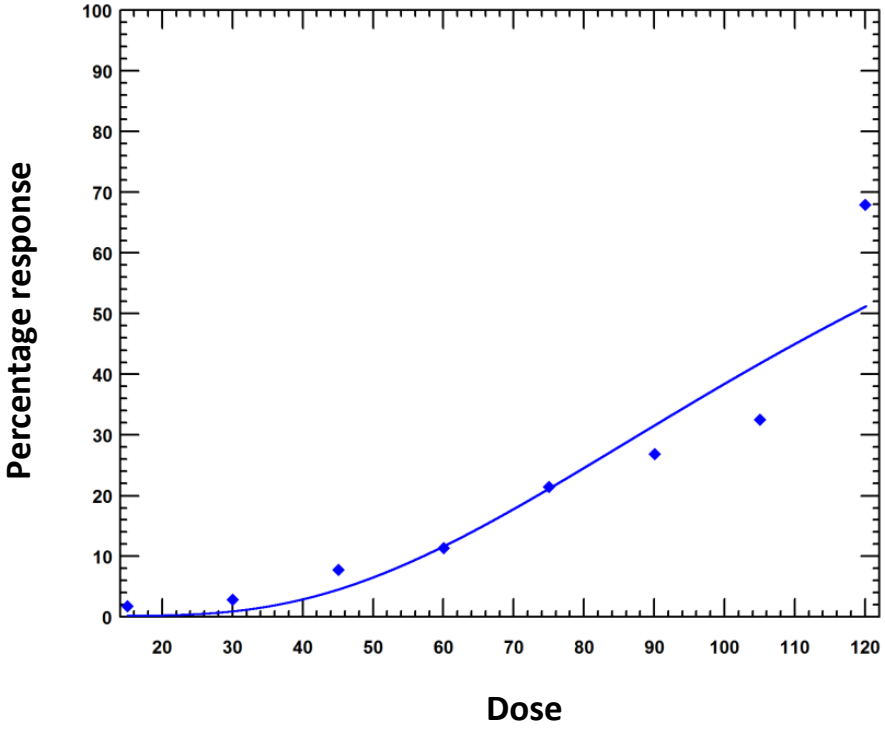

Figure 2. PoloPlus regression line showing percent response (=mortality) of *An. farauti* to C8910+silicone oil at 30 min

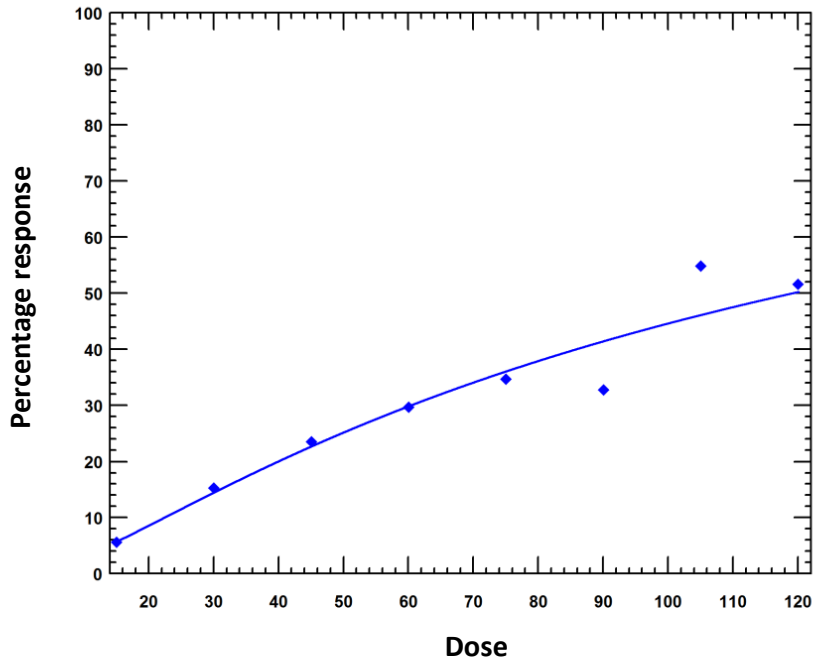

Figure 3. PoloPlus regression line showing percent response (=mortality) of *An. freeborni* to C8910+silicone oil at 30 min.

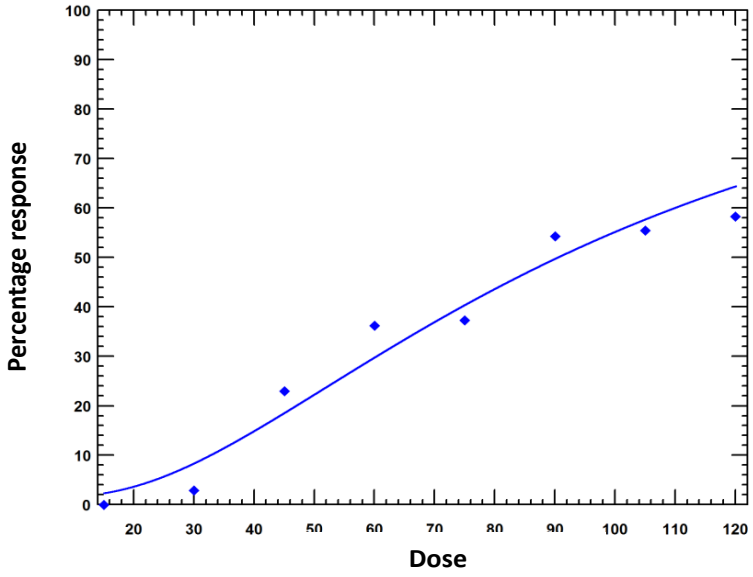

Figure 4. PoloPlus regression line showing percent response (=mortality) of *An. gambiae* to C8910+silicone oil at 30 min.

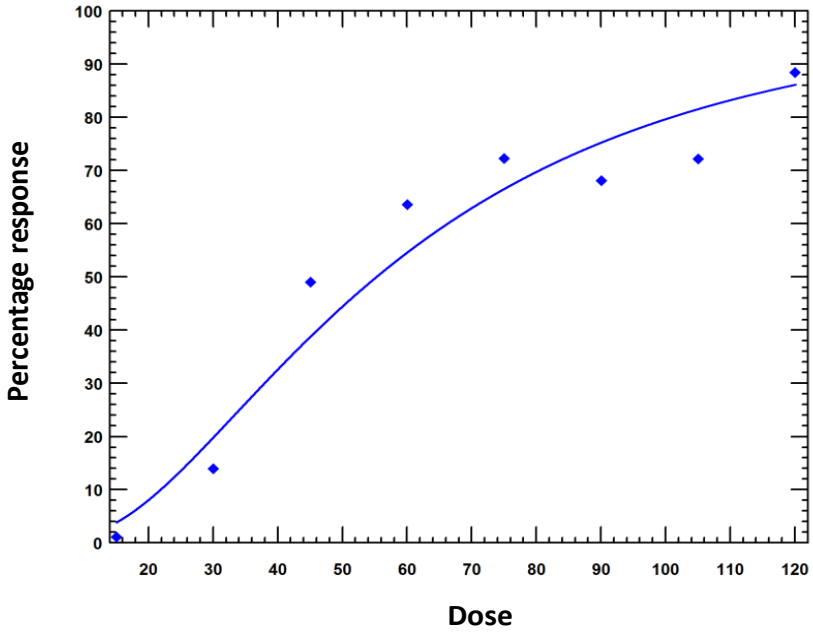

Figure 5. PoloPlus regression line showing percent response (=mortality) of *An. minimus* to C8910 at 30 min.

There is no apparent degradation of efficacy when C8910 is stored at room temperature for long periods of time (Dunford and Reifenrath, personal observations). However, further laboratory studies as well as field studies will be required to evaluate the residual efficacy of C8910. Because the fatty acids are semi-volatile organic compounds, it is thought that controlled release of the compounds would prolong their residual activity. Preliminary bioavailability evaluations using microencapsulated C8910 against *Ae. aegypti*, *Musca domestica* L.,

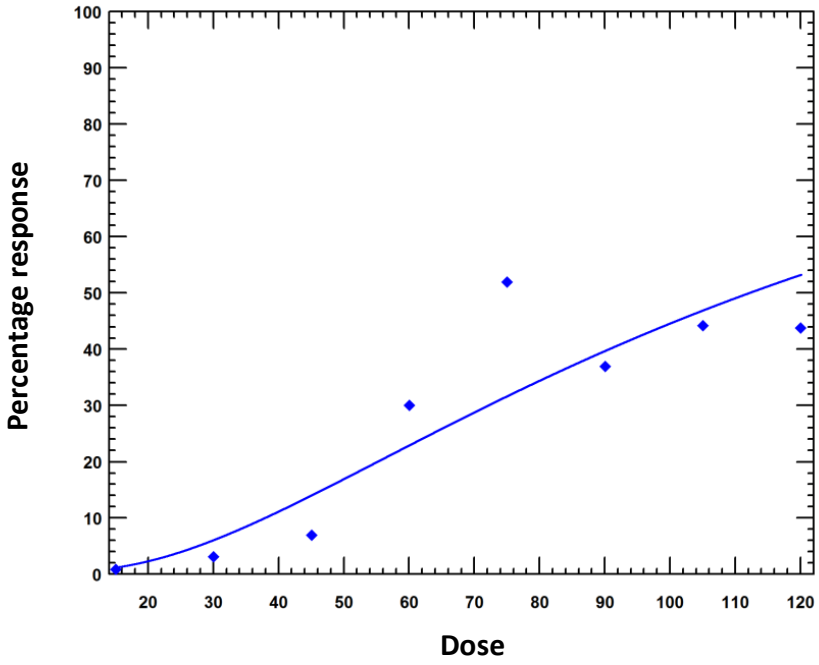

Figure 6. PoloPlus regression line showing percent response (=mortality) of *An. stephensi* to C8910+silicone oil at 30 min.

and *Blatella germanica* (L.) on treated filter paper showed significant mortality against *Ae. aegypti* at doses of 43.1 µg/cm^2 at 24 h, while house flies (431 µg/cm^2) and cockroaches were more resistant to the encapsulated C8910 (Reifenrath, unpublished data). Residual studies using controlled release C8910 formulations need to be further addressed.

The fatty acids comprising C8910 have been approved by the US FDA as food additives, suggesting they have low mammalian toxicity, an important quality for novel insecticides and probability they will pass current EPA (Environmental Protection Agency) regulatory statutes. Environmentally, the fatty acids are relatively benign, with low toxicity to bees and other beneficial insects (Reifenrath, 2001); however, this should be investigated further. When comparing our results with other WHOPES-approved insecticides against *Anopheles* species, concentrations of the C8910 formulation used herein required roughly 11 to 98 times (various pyrethroids) to 1.5 to 12 times (DDT) the diagnostic doses listed in Brogdon and Chan (2010) to achieve comparable mortality percentages; however, higher concentrations should be tested and may prove comparable to other insecticides. An alternative testing method, such as the WHO tube assay, should also be used to further determine if mosquitoes die after initial exposure and knockdown. Further development of C8910 formulations and associated production costs should be compared to traditional insecticides if products containing C8910 are used in the field for IRS (indoor residual spray) and/or treating bed nets.

C8910 is also being further developed for repellency attributes. It can be formulated for direct use on the skin, and topical formulations are being advanced for livestock use in the U.S. and for human use in South Africa. The minimum effective dose (MED) for C8910 to repel mosquitoes is estimated at a surface dose of 25 µg/cm^2, which is well above the LCD_{90} for all *Anopheles* species tested in Table 1. Since toxicity is dependent on contact, higher doses typically used for topical repellency (for example, 300 µg/cm^2) may exert mosquito toxicity only after the repellent dose decays below the MED. The repellency aspect of C8910 may not only provide a novel topical repellent, but it may also be a viable synergist to enhance other chemicals currently being used on treated bed nets. Synergistic interactions between insecticides and repellents have been shown to increase the residual life of impregnated materials and improve the control of pyrethroid-resistant mosquitoes (Pennetier et al., 2007). Pennetier et al. (2007) demonstrated a significant increase in residual efficacy by combining the repellents deet and KBR 3023 with pirimiphos methyl against *An. gambiae*. An earlier study also suggested a significantly higher efficacy was attributed to the combination of deet

with propoxur against pyrethroid-resistant *Ae. Aegypti* (Pennetier, 2005).

At present, the mode of action for C8910 has not been investigated, although preliminary studies suggest it is a respiratory inhibitor. The saturated fatty acids are seemingly simple organic compounds but have complex biological effects (Rioux and Legrand, 2007). Fatty acids have been shown to decrease respiratory activity and to uncouple oxidation from phosphorylation in isolated tissue preparations (Scholefield, 1963). Respiratory inhibition increased 2.5 fold for each carbon atom increase in chain length up to a maximum of 12 carbons (lauric acid). A further increase in carbon chain length results in a corresponding decrease in inhibitory activity. This inhibitory effect could be the result of surface tension effects on cell membranes as well as direct effects on enzyme systems. Insects that are sensitive to the toxic effect of low doses of C8910 tend to be repelled at higher doses, while other insects are insensitive to both repellent and toxic effects (Reifenrath, unpublished data). However, further research will be necessary to determine if the reactions are mechanistically related. C8910 shows promising characteristics for use in public health vector control. Future research directions involving C8910 should include the following: 1) description of the mode of action, 2) efficacy testing using stronger concentrations, 3) testing against resistant and other non-resistant mosquito species, to include wild populations, 4) determination of residual duration of controlled release C8910 and mortality results on building surfaces and fabrics, 5) potential use as a repellent against malaria vectors, 6) efficacy testing against other medically important arthropods such as filth flies, biting flies, fleas, kissing bugs, bed bugs, lice, and ticks, and 7) negative effects on non-target insects, birds, and mammals. Further development of C8910 and similar compounds may provide vector control specialists effective, environmentally-safe insecticides for controlling arthropod disease vectors, as well as a sustainable alternative to a growing insecticide resistance crisis.

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Conflict Interests

The authors declare that there is no conflict of interests

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Short Communication

Biodiversity and ecology of Culicidae and Simuliidae probable vectors of infectious diseases in villages of the Sanaga mid valley, Cameroon: Influence of the Sanaga River

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Most riverine villages of the Sanaga river are known endemic for vector-borne diseases. Two cross sectional surveys were set during two seasons in villages of the Sanaga mid valley to identify main Simuliids and mosquitoes genus and species, their specific biotopes and fluctuations of their abundance with respect to distance from the river banks and seasons. The study villages are located close to 5 and 35 km from the Sanaga river edges. Both larva and adult stages were assessed using known methods. All adults Simuliids, larvae and nymphs were identified as *Simulium damnosum*. Larvae and nymphs were collected only in the river stream and adults near the banks, farms and near households. Adults and larvae abundance was greater in the rainy season whereas nymphs were more abundant in the dry season. Endophilic mosquitoes harvested were *Anopheles* and *Culex*. Their abundance was greater in villages close to the river. Species and resting densities varied with distance from the river edges. Culicidae larvae collected belonged to *Aedes*, *Anopheles* and *Culex*. Fourteen species were identified, 3 of *Anopheles*, 5 of *Aedes* and 6 of *Culex*. Some species showed broad specificity to biotopes. This study indicates that mosquito fauna is more diversified at larval stage in the Sanaga mid valley; some having broad specificity to breading sites. The Sanaga river harbours most of the species found in the water bodies. Indoor adult mosquitoes are less diversified indicating that most of the mosquito found at larval stage may breed mostly outdoor. *Simulium* larvae and pupae breed specifically in the falls and rapids of the Sanaga stream. Both sexes of adult *Simulium* are found near the river, whereas only females are found near households and in farms. Adults *Simulium* density decreases with the distance from the river with two picks of abundance in the day.

Key words: Biodiversity, Culicidae, Simuliidae, Sanaga River.

INTRODUCTION

Insect borne diseases are most harmful in social economic importance among endemic diseases in most tropical areas. Transmission of these diseases is mostly related to insect's adult flies among which mosquitoes

(Culicidae) and black flies (Simuliidae) families are believed to be most important. Culicidae or mosquitoes are known to transmit either virus, bacterial or parasitic diseases, whereas Simuliidae or black flies are known as specific vectors of onchocerciasis in tropical areas (Rodhain and Perez, 1985; Rodhain, 1999; Ostfeld and Keesing, 2000).

The Sanaga mid valley is an area where despite mass distribution of ivermectin, the onchocerciasis-specific treatment, onchocerciasis is still highly endemic. Foremost, onchocerciasis endemicity level in this area is likely to favour the occurrence of epilepsy, a neurologic disease. Previous studies demonstrated that neighbouring villages of the Sanaga river are mesoendemic to hyperendemic for malaria (Gazin et al., 1989).

The Sanaga river is of most importance in Cameroon. The streams are mostly fast flowing with many rapids and falls on rocky substratum suitable as breeding sites for *Simulium* (Mouchet, 1962; Philippon, 1977; Same-Ekobo, 1997). Apart from favouring development of *Simulium* larva, the river can be expected to favour development of other flies like mosquitoes, thus influencing epidemiology of other vector borned transmitting diseases.

A descriptive study was done in the diversity and spatial distribution of potent endemic diseases transmitting Culicidae and Simuliidae flies in the Sanaga river and in three villages situated at different distances from the river edges in the Sanaga river mid valley. Studied villages were Mbebe, Ndomnjengue and Bot Makak.

The main objective of the study was to investigate species that can be found in the study villages with respect to distance from the Sanaga river edges and specify ecological peculiarities of species found in prospected areas.

MATERIALS AND METHODS

Study area

The Sanaga mid valley is located in a forest-savannah transition area covering almost 150 km distance between the Monatele town in the Lekie division upstream and Edea town in the Sanaga Maritime division downstream. This valley covers three divisions, namely, Lekie, Nyong-Ekelle and Sanaga-Maritime divisions. The Sanaga river is marked at this level by existence of rapids and one of it most important falls; the Mbebe-Kikot falls with an almost rocky substratum.

The studied villages were Ndomdjengue located in the Sanaga-Maritime division 5 km from the main Mbebe-Kikot falls of Sanaga, village Mbebe located close to the edges of the Sanaga river and close to the later falls of the area in the Nyong-Ekelle division and the village Bot-Makak located 35 km from the river edge in the Nyong-Ekelle division. Ndomdjengue and Mbebe villages are located in forest-like areas, whereas Bot-Makak is a small town surrounded by a forest area. Moreover, Ndomdjengue village is separated from the river by an evergreen forestry screen. Farming, bovine rearing and commerce are the main occupations in the three villages. Fishing is also made by residents in Mbebe. This study consisted in collecting and identifying adults as well as larvae stages of *Simulium* and Culicidae. Sampling techniques and conservation varied according to insect groups and stages.

Simuliidae collection and preservation

Adult *Simulium* were captured using aluminium panel trap (100×100×0.55 cm) coiled with glue made of Tween 20 and 95° alcohol. The trap was set from 6 am to 6 pm at 2 m from the ground and flies caught at each hour were collected, counted and transferred into tubes. These tubes were then closed with a dry cotton wool and brought to laboratory for identification. The traps were set in farms, near habitations, at the river edges and in the forest undergrowth.

Larvae and pupae of *Simulium* were collected in the stream of the river on rocky substratum, and in the falls from leaves and stems of submerged and floating vegetation. Soft forceps and plastic pipettes were used to collect larvae on rocky substratum. Larvae hanged on submerged and floating vegetations were collected with soft pipettes after tearing the vegetation using a hook. Larvae and pupae collection lasted 30 min at each site. Larva stages were then transferred in test tubes containing 70° ethanol, and then carried to laboratory for identification.

Culicidae collection and preservation

Indoor resting adult mosquitoes were harvested in each village through pyrethrum insecticide spray in a room of known dimensions and where a 2×2 m white sheet was previously placed. The spraying took place between 10 pm and midnight. Dead and alive mosquitoes collected on the white sheet were transferred into test tubes, and then brought to the laboratory for identification. Indoor-resting density for adults Culicidae was calculated with the following formula: Total number of adults collected/Number of rooms screened.

Larva and pupa of mosquitoes were searched in different water bodies across the villages, namely, Sanaga river falls, rapids, marshes, pools, ponds, springs, holes in trees, abandoned containers like flasks, tyres, and dishes. Culicidae larvae and pupae were collected in large water bodies using the classical “dipping” technique as described by Service (1976). In small water bodies like abandoned containers or tyres and tree holes, larva stages were collected using a plastic pipette. All larva collected were brought to the laboratory in flasks containing water from the collecting site for identification.

Some of the mosquito larvae were bred in laboratory for adult emergency for accurate identification. The larvae were fed in plastic containers until adult harvest with organic detritus from water collected in their natural breeding sites. These adults were then identified using morphological keys.

Identification of harvested insects

Both Culicidae and Simuliidae adult and larval stages were

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examined under a stereomicroscope and identified using the specific morphological characteristics identification keys for *Anophelinae* species (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987), Culicinae (Jupp, 1996) and Simuliidae (Freeman and De Meillon, 1953). Data were analysed using Chi^2 test at confidence interval of 0.05.

RESULTS

Biodiversity and abundance of Simuliidae

A total of 996 adults *Simulium* (black flies) were collected in the villages with 90.7% in May (rainy season) and 9.3% in December (dry season). All adult black flies were identified as *Simulium damnosum* Theobalt. Abundance of adult stages decreased significantly with distance from the Sanaga river edges with adult *Simulium* collected, 819 (53.2%) were harvested at Mbebe, 166 (43.9%) at Ndomnjengue and 11 (2.9%) at Bot Makak.

Adult black flies abundance varies also within the same village with distance from the breeding site. At Mbebe for example, of 766 adult black flies caught in May (rainy season), 618 (80.7%) were caught at the vicinity of rapids in the Sanaga river and the remaining far from the breeding site including 97 (12.7%) near households and 51 (6.7%) in farms.

Abundance of adult black flies also showed variation with seasons. In fact, 378 adult *Simulium* were harvested close to households and farms. 93 (24.6%) of them were collected in December and 285 (75.4%) caught in the rainy season (May). The difference recorded is statistically significant between the two seasons ($p<0.001$) indicating that the rainy season may favour the spread of adult black flies throughout the farms and near human habitats.

Daily cycle of adult *Simulium* flies

Hour to hour harvesting of adult black flies from 6 am to 6 pm allow identification of two peaks of maximal activities of adult flies, with one in the morning between 8 and 9 am (20% of adults caught) and the second in the late afternoon between 5 and 6 pm. Flies were less abundant between 1 and 3 am with an aggregate 2% of adult *Simulium* caught during the 2 h.

Abundance variation between households and farms

In all villages screened, adult black flies were found mostly near households than in farms with differences being statistically significant. In fact, adult *Simulium* caught outside from the Sanaga river edges, 65.5, 100 and 50.7% were caught near households at Mbebe, Bot Makak and Ndomdjengue, respectively. The remaining was captured in farms from the other village.

Distribution of adult *Simulium* flies according to sex in the screened areas

Of the adult *Simulium* caught on traps, males were caught only along the Sanaga river edges and Mbebe, whereas only female black flies were caught in the other villages near households far from the river edges and farms. Of the adult *Simulium* harvested at Mbebe, 44 (7%) were males and 574 (93%) females.

Larvae and pupae

Simulium larvae and pupae were found only in the Sanaga River mostly hanged on submerged aquatic rocky substratum and vegetables. This submerged and floating vegetation was identified to family Podostemaceae, namely, *Dicraeananthus africanus*.

Larva and pupa were mostly abundant in the rainy season. In fact, of *Simulium* larvae and pupae harvested in the two seasons, 69.4% of the larvae and 52% of the pupae were caught in the rainy season. All the larvae and pupae were identified as *S. damnosum* complex. The difference found between abundances of larvae collected is statistically significant between the two seasons with the rainy season being more suitable for development of these stages ($p<0.001$), whereas this difference is not statistically significant among pupa harvested in either season ($p>0.30$).

Culicidae species diversity in the study area

Forty species were identified in this sample. Three species were found among mosquitoes of the genus *Anopheles*: *Anopheles gambiae*, *Anopheles nili*, and *Anopheles funestus*. Those of the genus *Aedes* belonged to five species, namely, *Aedes aegypti*, *Aedes vittatus*, *Aedes chaussieri*, *Aedes ledgeri*, and *Aedes albopictus*. Six species were found in the genus *Culex*, *Culex quinquefasciatus*, *Culex perfuscus*, *Culex rubinotus*, *Culex chorleyi*, *Culex simpsoni*, and *Culex insignis*.

Species diversity in each village

The number of Culicidae spp. and development stages varied among villages. Thus, *A. gambiae*, *A. aegypti* and *C. quinquefasciatus* were harvested in the three villages visited, while *A. ledgeri* was harvested only at Ndomdjengue and Bot Makak. Species *A. nili* and *A. funestus* were found at Mbebe and Ndomdjengue. Species *A. Albopictus* and *C. simpsoni* were found only at Bot Makak. Species *A. vittatus*, *A. chaussieri*, and *C. Insignis* were harvested only at Mbebe whereas *C. perfuscus*, *C. rubinotus*, and *C. chorleyi* were collected only at Ndomnjengue.

Stage-related diversity

Of Culicidae spp. harvested, *A. gambiae* and *C. quinquefasciatus* were found either as adult or larval stages, whereas *A. nili* and *A. funestus* were found only as adult stage. The other ten mosquito species were found only as larval stage.

Mosquitoes of the genus *Aedes* were found only at larval stages in our sampling. Five species were identified among specimen harvested including: *A. aegypti* (88.9%), *A. vittatus* (2.6%), *A. chaussieri* (1.1%), *A. ledgeri* (2.3%) and *A. albopictus* (5.1%).

Six species were identified among mosquitoes of the genus *Culex*, namely, *Culex quinquefasciatus* (16.2%), *C. perfuscus* (1.6%), *C. rubinotus* (52.7%), *C. chorleyi* (15.1%), *C. simpsoni* (4%) and *C. insignis* (10.4%). *C. quinquefasciatus* was the only species found at adult stage, the others were found at larval stage.

Culicidae larva stages in breeding sites

Thirty four (34) breeding sites were screened: 9 (26.47%) at Ndomnjengue, 6 (17.65%) at Bot-Makak, 19 (55.9%) at Mbebe. Of those screened at Mbebe, 78.95% were located at the edge of the Sanaga river and the other away from the edges, so the rocky banks of the Sanaga river at Mbebe seem propitious for development of mosquito larva.

Of the larvae collected, 857 (63%) were collected at Mbebe, 371 (27.25%) collected at Ndomnjengue, and 133 (9.75%) collected at Bot Makak. Culicidae larvae were harvested in a variety of breeding sites: permanent, temporary and natural water bodies.

Mosquito larvae from Ndomnjengue belonged to the genera *Aedes* and *Culex*. Of the total number collected in this village, those of the genus *Aedes* belonged to two species, namely, *A. aegypti* (16.2%) and *A. ledgeri* (2.9%). Four species were identified among those of the genus *Culex*, namely, *C. quinquefasciatus* (5.65%), *C. perfuscus* (1.9%), *C. rubinotus* (61.2%) and *C. chorleyi* (12.1%). No *Anopheles* larva was found in this village.

At Mbebe, *A. gambiae* was the only Anopheline larva collected. This species represented 8.75% of the sample collected in this village. Mosquitoes of the genus *Aedes* belonged to three species including *A. aegypti*, *A. vittatus*, and *A. chaussieri* accounting for 75.95, 2.6 and 1%, respectively of the sample collected in this village. Culex larvae belonged to three species: *C. quinquefasciatus*, *C. insignis*, and *C. Chorleyi.* These Culicidae spp. represented 4.1, 5.2 and 2.3% of the total Culicidae larvae collected as in Table 1.

At Bot Makak, no *Anopheles* larva was found. Mosquitoe larvae collected were *Aedes* and *Culex*. *Aedes* larvae were of three species: *A. aegypti*, *A. ledgeri* and *A. albopictus* representing 38.3, 6.8 and 33.1% of the specimen collected, respectively. Culex larvae were of two species: *C. quinquefasciatus* and *C. simpsoni* comprising for 9 and 12.8% of Culicidae larvae collected in this area.

Distribution of mosquito larvae in breeding sites

A. aegypti was found in natural temporary as well as permanent breeding sites notably in abandoned water-storage containers near households and holes in trees. This species was usually found together with one or more of the following species: *C. rubinotus*, *C. perfuscus*, *A. albopictus*, *A. ledgeri* and *C. simpsoni*.

Species *A. aegypti* and *C. quinquefasciatus* were found in the three villages, while *A. gambiae*, *A. vittatus*, and *A. chaussieri* were present only at Mbebe, and species *A. albopictus* and *C. simpsoni* collected only at Ndomnjengue. Species *A. gambiae*, *A. vittatus*, *A. chaussieri*, and *C. insignis* were found mostly in clean water contained in holes on the rocky substratum of the Sanaga river edges.

C. quinquefasciatus specimen were found in water bodies bearing organic materials in permanent ponds of the undergrowth forest at Ndomdjengue, and in clean water bodies with holes of rocky substratum of the Sanaga banks.

Of the 8 larvae of mosquito species found at Mbebe, 6 (75%) were harvested in the Sanaga river and the 2 (25%) outside.

Spatial distribution of adult *Culicidae spp.*

At Ndomdjengue, 67.7% of the Anophelines collected were identified as *A. gambiae*, 25.8% as *A. nili* and 6.5% as *A. funestus*. *Culex* and *Aedes* mosquitoes were not harvested at adult stage in this village. At Mbebe, *A. nili* specimen accounted for 55.6% of the Anohelines collected, *A. gambiae* for 40.7 and 3.7% were *A. funestus*. *Culex* and *Aedes* mosquitoes were not harvested at adult stage in this village. At Bot-Makak, 86.7% of the adult mosquito specimens were *A. gambiae* and 13.3% were *C. quinquefasciatus.* Adult *Aedes* was not found in this area.

Therefore, three *Anopheles* species, namely, *A. gambiae*, *A. nili* and *A. funestus* were found in the two villages close to the Sanaga River (Mbebe and Ndomnjengue), whereas *A. gambiae* is the species found at Bot Makak.

Of the mosquitoes harvested in the three villages, *A. gambiae* specimen account for 46.7, 24.4 and 28.9% at Ndomnjengue, Mbebe and Bot Makak, respectively. The species *A. nili* and *A. funestus* found only at Ndomnjengue and Mbebe representing 34.8 and 66.7%, respectively in the first village, 65.2 and 33.3% at Mbebe.

Adult mosquitoes resting densities

Average resting densities of mosquitoes recorded in the villages were 1.5, 1.48 and 0.65 at Mbebe, Ndomnjengue and Bot-Makak, respectively.

Considering mosquito species, resting densities for *A. gambiae*, *A. nili*, *A. funestus* and *C. quinquefasciatus* are 0.73, 0.37, 0.05 and 0.03 in the three villages. The highest resting densities per species are 1.00 for *A. gambiae* at Ndomdjengue, 0.83 for *A. nili* at Mbebe, 0.1 for *A. funestus* at Ndomdjengue and 0.09 *C. quinquefasciatus* at Bot Makak.

DISCUSSION

Culicidae and Simuliidae are leading transmitters of vector borne diseases in the tropics. Their abundance is mainly influenced by the existence of water bodies for larvae breeding. However, occurrence and spread of chemoresistance to pathogens as well as the vectors is the main difficulty to effective control of these diseases. Vector control is becoming more difficult due to the complexity of vectorial system in addition to chemo-resistance, since some strains or species are becoming more susceptible to insecticides than others. The complexity of the vectorial systems become harmful with the zooanthropophilic capacity of some insects vectors which feed both on human and livestock, thus facilitating transportation of the pathogens to human neighbourhood. The launching and implementation by the National Malaria Control Program of the prevention of disease transmission through fighting vectors intend to eliminate or lower abundance of potent anthrophilic insects that feed on human being.

This study was a first step on the estimation of the risk of vector borne diseases outbreaks or persistence in villages situated at different distances from the Sanaga river in a forest/savannah area and to assess the influence of this river on the biodiversity, the spatial distribution of major Culicidae and Simuliidae species among investigated habitats in the Sanaga mid valley.

Fourteen species were identified among Culicidae larvae from the 34 potent larval breeding sites investiga-ted from the Sanaga river banks to Bot Makak area situated at 35 km from river edges. Most of these species have been mentioned in previous studies on mosquito fauna in Cameroon (Rageau and Adam, 1952; Rickenbach et al., 1976a, b; Fontenille and Toto, 2001; Awono-Ambene et al., 2004). The Sanaga river banks seemed more suitable for the development of mosquito larvae, since these banks harboured six of the eight larvae Culicidae spp. found in village Mbebe the closest village. The river banks may thus offer better physical and chemical conditions for larval stages development. This finding corroborates reports from the Comoe River in Ivory Coast which bears more larval mosquito species than other water bodies in the village (Adja et al., 2006).

Mosquito species were found more diversified at larval stage and also showed broad specificity for breeding sites. *A. aegypti* and C*. quinquefasciatus* larvae were found in all areas screened indicating its ubiquity as previously reported in Cameroon (Rageau and Adam, 1952, 1953; Rickenbach et al., 1976a). However, the presence of C*. quinquefasciatus* larvae in all water bodies types, both sunny and shady sites, clear and organic materials bearing water bodies, though similar to reports from Cap Vert (Larivière and Abonnenc, 1958), contrast with earlier data reporting *C.* species to prefer organic materials bearing water bodies in urban settings of Africa (Subra, 1973; Robert et al., 1986; Hougard et al., 1993).

C. perfuscus and *A. ledgeri* larvae showed a broad specificity to tree holes bearing water bodies, while *A. vittatus* larvae specific habitats were holes on stones along the Sanaga river banks. This study is a first demonstration of *A. vittatus* larvae in the Sanaga mid valley. Demonstration of *A. ledgeri* in Cameroon is a new observation. *A. albopictus* larvae showed microhabitat specificity to small size natural and artificial water bodies mostly abandoned tyres and containers corroborating previous observations in Cameroon (Fontenille and Toto, 2001), though this species seemed not largely distributed as previously thought. Further investigations throughout the four seasons in this area will enable conclusion of this findings.

The species *C. rubinotus* found at Ndomdjengue has already been suspected in Cameroon, but the authors did not confirm this species in Cameroon since it was known as an Asian species (Rageau and Adam, 1952). *C. rubinotus* has already been identified in the Culicids fauna of Gabon (Service, 1976; Mouchet, 1971), a border country with Cameroon enables the confirmation of these findings which indicates that this *C. rubinotus* may be migrating upward from Gabon.

The presence of *A. nili* and *A. funestus* adult specimen and their absence at larval stages in our sampling may be due to the heavy rainfall before our arrival in the area which washed of larval breeding sites. Previous studies in Cameroon reported *A. nili* and *A. funestus* larval stages usually attached to aquatic plants (Le Goff at al., 1990; Huang, 2004).

The results of this study are of medical importance. Demonstration of *Aedes* species indicates a potent for arboviruses transmission in Cameroon (Huang, 2004; Cattand et al., 2006). *Anopheles* spp. found in this study are foremost of malaria vectors system in Cameroon (Fontenille and Lochouarn, 1999; Carnevale et al., 1992). The simultaneous existence of three species at adult as well as larval stages may favour permanent transmission of malaria and other parasitic disease like lymphatic filariasis in the area. A previous parasitological study in village demonstrated high prevalence rates for malaria and onchocerciasis (Gazin et al., 1989). These high Onchocerciasis high prevalence rates in the area indicate

Table 1. Larvae and adult Culicidae species distribution in the study area.

Species	Mbebe		Ndomdjengue		Bot-Makak	
	Larvae	Adult	Larvae	Adult	Larvae	Adult
A. Gambiae	2	Present	0	Present	0	Present
A. Nili	0	Present	0	Present	0	Absent
A. Funestus	0	Present	0	Present	0	Absent
A. aegypti	4	Absent	3, 4, 5	Absent	4, 5	Absent
A. vittatus	2	Absent	0	Absent	0	Absent
A. chaussieri	2	Absent	0	Absent	0	Absent
A. ledgeri	0	Absent	5	Absent	5	Absent
A. albopictus	0	Absent	0	Absent	4, 5	Absent
C. quinquefasciatus	2	Absent	3	Absent	1	Present
C. perfuscus	0	Absent	4	Absent	0	Absent
C. rubinotus	0	Absent	5	Absent	0	Absent
C. chorleyi	3	Absent	3, 4	Absent	0	Absent
C. simpsoni	2	Absent	0	Absent	5	Absent
C. insignis	2	Absent	0	Absent	0	Absent

Larvae microhabitats: 0, Absent; 1, ponds with clear water; 2, holes in rocky substratum in the Sanaga river; 3, ponds bearing organic materials in the forest undergrowth; 4, containers near households and farms; 5, holes in trees bearing organic materials.

a good efficiency of *S. damnosum* in the transmission.

Studies on the epidemiology of arboviruses in Cameroon are scarce and we can not comment on the efficiency of the potent vector in the transmission of these pathogens in the area

Indoor Pyrethrum spraying sampling technique collected only female Culicidae with *Anopheles* specimen being most abundant. This abundance of Anophelines upon *Culex* and *Aedes* has already been demonstrated in previous studies at Ndomnjengue and Mbebe using joint capture with indoor spraying and mosquito net (Le Goff et al., 1990, 1994; Huang, 2004; Carnevale et al., 1992). Adult *A. nili* were harvested at Ndomnjengue more than 1.5 km from the larvae breeding sites in the Sanaga river banks at Mbebe, indicating that adult *A .nili* dispersal capacity is greater than thae stated in previous studies (Le Goff et al., 1990). However, adult *A .nili* do not reach farther distances as indicated by their absence at Bot-Makak (35 km from the Sanaga river).

Rapid decrease in indoor resting densities of adult *A. nili* and *A. gambiae* with distance from the river edges is indicative of the influence of the Sanaga river stream. This influence of the Sanaga river is also indicated by the distribution of larvae breeding which shows *A. nili* larvae mostly located in water bodies on the left hand of the Sanaga river under vegetations, whereas *A. gambiae* larvae were mostly harvested on the right hand. The dense vegetation which separates the two arms may force adult *A. nili* to fly towards the Mbebe area and *A. gambiae* to go mostly towards Ndomdjengue.

The rapids and falls at Mbebe-Kikot are found to be the main *Simulium* larvae breeding sites in the study areas. At this level, stream turbulence favours optimal development of *Simulium* larvae and pupae. Absence of larvae breeding sites downstream may be due to smooth flow of the stream. Larvae and pupae were collected only on immerged vegetation. Their absence on rocky substratum may be due to the presence of algae overcastting on rocks. Such observations have already been made in Ghana where the presence of algae on rocky substratum was thought to limit development of *Simulium* larval stages (Opoku, 2006).

Only *S. damnosum* larvae and pupae were found in our sample. Further studies based on monthly collection during a year period need to be undertaken to identify the presence of other species that may compete with *S. damnosum*. In river Pra in Ghana, *S. damnosum* was shown to dominate over *S. adersi* and *S. unicornutum* (Opoku, 2006). Such competitive domination by *S. damnosum* has also been demonstrated in river Maraoue in Ivory Coast against *S. adersi* and *S. tridens* (Elouard and Gibon, 1985). However, the results of this study are consistent with previous studies in the mid Sanaga which demonstrated *Simulium squamosum* (s.s) to be the main species of the *S. damnosum* complex in this area (Traore-Lamizana and Lemasson, 1987; Traore-Lamizana et al., 2001). Our results have extrapolated to the mid Sanaga valley, but absence of respiratory filaments in pupae collected in our sample may be indicative of particular strain of *S. squamosum*. In previous

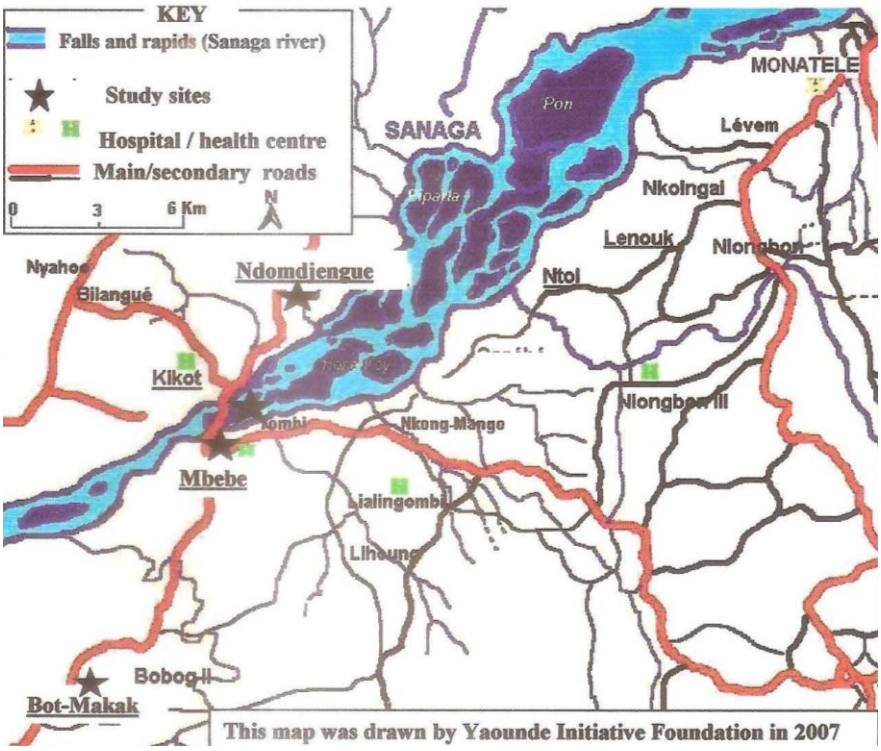

Figure 1. Map of the study area.

previous studies, the species found in this area was described as "form B" of *S. squamosum* (Traore-Lamizana et al., 2001).

Larvae and pupae were found more abundant in the rainy season (May) than the dry season (December). This seasonal increase can be explained by the increase of flow of the river which also leads to an important increase of water turbulence thus improving the nutritional status of larvae (Opoku, 2006). Pupae abundances were not significantly affected by season alternation, indicating that increase in larvae abundance does not systematically influence pupae stage formation. Therefore, larvae stage may extend in the rainy season to favour constant pupae densities as demonstrated in Ivory Coast where temperature fall is demonstrated to slow down the development of *Simulium* immature stages (Bellec and Hebrard, 1983). The constant abundance of *Simulium* pupae throughout seasons leads to permanent emergence of adult through the year in the rapids and falls of Mbebe-Kikot.

Adults *Simulium* specimen were found more abundant in the rainy season than the dry season. Females had a greater dispersal capacity than males. Adult males were caught only close to the breeding sites at Mbebe whereas female reached 35 km from the larval breeding sites. These females showed greater abundance all along the day in areas mostly visited by human like households vicinity; farms indicating man biting rates may be important and transmission of related filarial continuous in our study area as shown in Figure 1.

The circadian rhythm of adult black flies in the villages in the rainy season indicate two picks one early in the morning (8 to 9 am) and the second late in the evening (5 to 6 pm). Between the two peaks, adult flies abundances was weak, indicating that *Simulium* flies are less active during hot periods of the day and very active during cooler hours. This bimodal in the *Simulium* daily activity are closed to findings in the Soudanian savannah area in Mali (Western Africa) where a peak was recorded from 8 to 11 am and the second in the evening ((Bellec and Hebrard, 1983).

Abundance of adult *Simulium* was greater near households than farms in all villages, indicating high nuisance by black flies near households than in farms. This finding may be due to the existence of a chemical attraction by man vis-à-vis of flies which need to feed on human being. This attractive effect can be justified at Ndomdjengue which is a savannah area where habitants are mostly farmers and adult flies were found at similar abundance near households as well as in farms. In forest areas like Mbebe, human occupations take place mostly close to households whereas the working zones can exist farther from households in the savannah areas for their occupational activities.

Demonstration of the diversity and widespread insect fauna in the Sanaga mid valley has epidemiological

implications since most of the species collected are major known vectors of endemic diseases in Cameroon and other countries. Anopheline species *A. gambiae* harvested in all the study areas, *A. nili* and *A. funestus* collected in villages close to the river edges, namely, Mbebe and Ndomnjengue are well known efficient malaria vectors in Cameroon (Antonio-Nkondjo et al., 2006). Simultaneous existence of the three species in villages close to the Sanaga river edges is relevant for amplifying the exposition risk to malaria parasites and other anopheline transmitted diseases.

Onchocerciasis in the study area is of social and economical importance since many residents suffer from sight impairment, blindness. Furthermore, pest due to *Simulium* flies is of economical importance in hampering farming for example. Nuisance due to *C. quinquefasciatus* flies are of same effect though this fly is not known for transmitting infectious disease in Cameroon. Lymphatic filariasis infections are reported in health centers record books, but specific pathogens are not yet found. However, the presence of *A. gambiae* and *A. funestus* known to be vectors of this filariasis in Cameroon is evocative of existence of lymphatic filariasis in the study area. Parasitological investigations are ongoing to assess filariasis endemicity in the area.

Knowledge on human arbovirosis is scarce in Cameroon. However, the presence of known yellow fever and dengue virus mosquitoes vectors in Cameroon and elsewhere in the world notably *A. aegypti* and *A. albopictus* in our study sample indicate a possible transmission risk of these pathogens in the study areas. Other mosquito species like *A. vittatus* and *C. rubinotus* potent vectors of arboviruses in other parts of Africa were found in our collections, though only at larval stages. Occurrence of *A. ledgeri* is however a new observation in Cameroon.

Conclusion

The mid Sanaga valley harbours a great variety of potent vectors of parasites and arboviruses. *Anopheles*, *Culex*, *Aedes* and *Simulium* species recorded in the Sanaga mid valley are of the man or animal biting-flies mostly involved in diseases transmission in Africa.

Culicidae larvae are widely distributed in the study area, some having broad specificity for their micro-habitats, whereas *Simulium* larvae are found only in the Sanaga river. Distribution of Culicidae larvae varies among villages with distance from the Sanaga river, this river harbours almost all Culicidae spp. recorded at larval stage. The Sanaga River thus influences largely the biodiversity of the entomological fauna in the mid Sanaga valley. Adult Culicidae spp. are less diversified than larvae. Their distribution also decreases with distance from the Sanaga river edges. Occurrence of *A. ledgeri* as well as *C. rubinotus* is however a new observation in Cameroon.

S. damnosum was the species found. Adult *Simulium* had a high dispersal capability reaching up to 35 km from the main breading site. Their daily activity had a bimodal dispersion with a peak in the morning and the second late in the evening.

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Conflict Interests

The authors declare that there is no conflict of interests.

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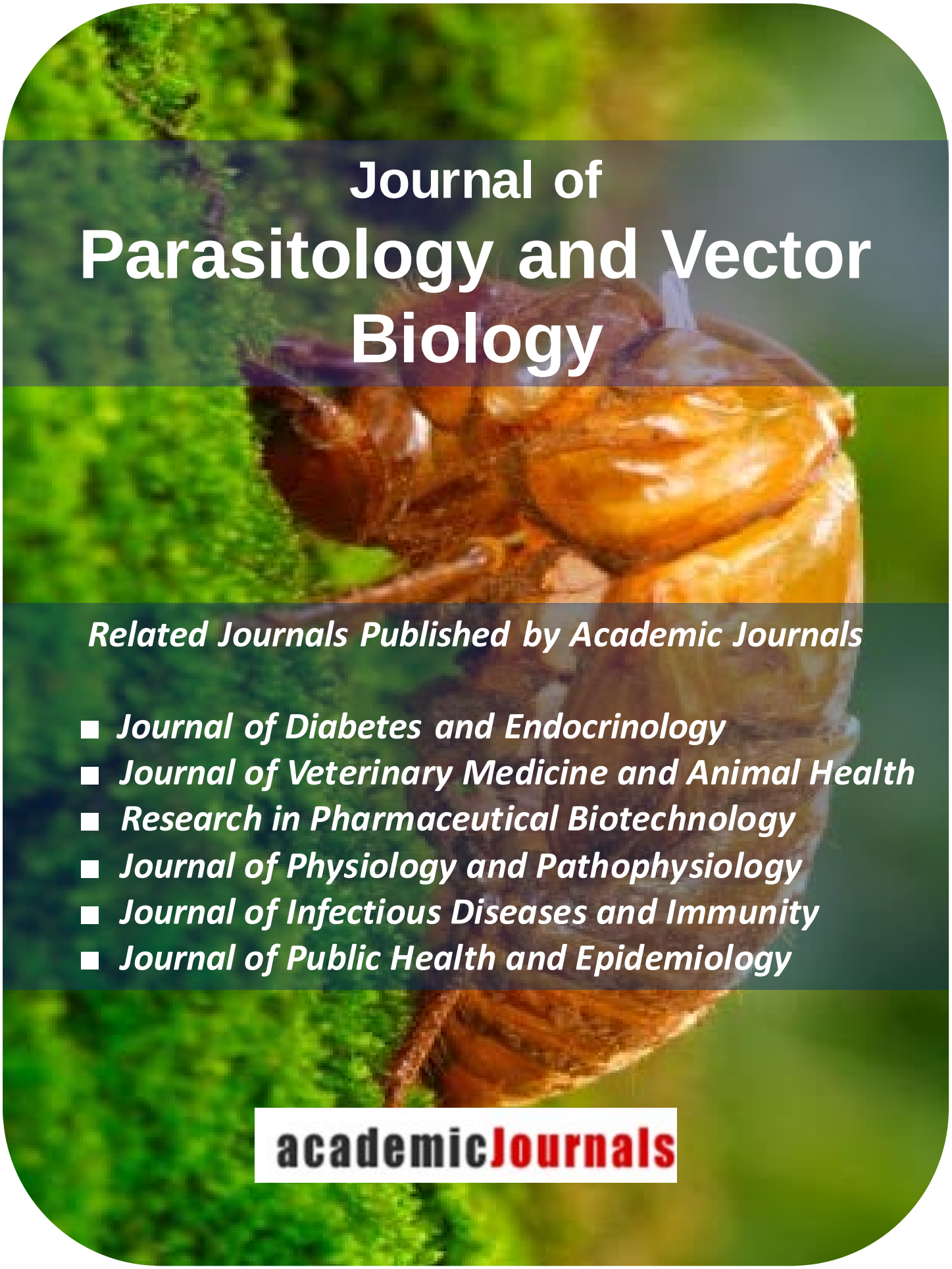

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