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Prevalence of *Anopheles Gambiae* S. S and their pyrethroid knock down resistance pattern in five selected communities in kumasi metropolis using polymerase chain reaction (PCR)

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ABSTRACTS

The Anopheles species play a central role in malaria transmission. Several methods are available to map out the prevalence of various species of the Anopheles and the knock down resistance in the tropics and sub-tropical regions of the world. PCR is faster in determining genetic diversity of these species. This study was set out to identify and determine the prevalence and the pyrethroid knock down resistance pattern of Anopheles gambiae s.s, (the most prevalent malaria vector) within the selected communities in Kumasi. DNA was extracted from Anopheles mosquitos' larvae and used as template in polymerase chain reaction (PCR), for each specimen. Seventy six out of 100 larvae collected in all the sites were identified as gambiae s.s. Twenty six out of 50 identified An. gambiae s.s were found to posses Kdr gene with remaining 24 samples being susceptible. PCR was successfully used for the identification and prevalence of An. gambiae s.s and determination of their pyrethroid knock down resistance.

INTRODUCTION

Malaria remains one of the most devastating diseases occurring in the world today. It is estimated that about 350-500 million clinical cases occur every year with approximately 1-3 million deaths in tropical Africa alone (1, 2). This represents at least one death in every thirty seconds (3). Majority of the cases occur in children under five years (2, 5) and pregnant women are also especially vulnerable. Approximately 40% of the world's population live in regions where malaria transmission is endemic (6).

In Ghana, malaria is one of the major public health problems. The disease is hyper-endemic and accounts for nearly 22% of all death and between 42 - 44% of all out- patients cases. This ranks fifth as the commonest cause of death in the 0-4 year age group (7).

It is estimated that, African economies spend as much as $12 billion every year fighting malaria and that is 1.3% of GDP annually lost (8).

In Ghana, the cost of treating malaria between 2007 and 2008 amounted to US $772.4 million. This amount that equalled to the Ghana's entire health budget for 2008 and 10% of the country's entire Gross Domestic Product for 2006 (9**).** Again in the 2009 budget an amount of GH¢921 million was allocated to the health sector, out of which nearly 90% is spent on malaria (7).

Anopheles mosquitoes are the principal vector of malaria transmission in the tropical and sub-tropical areas of the world (10). *Anopheles* belongs to the order *Diplera*, sub-order *Nematocera*, family *culidae* and sub-family

Anophelinae. The species have a worldwide distribution, occurring in both tropical and temperate regions (11). There are over five hundred known species of *Anopheles*, but only sixty are known to transmit malaria (11).

In Africa, most of the important malaria vectors belong to a species complex, whose members are difficult and sometimes impossible to distinguish morphologically (12, 13, and 14). These difficulties have stimulated the development of molecular tools for precise and reliable identification of sibling species. The study of the distribution of specific malaria vectors which was the focus of this work is required for meaningful planning and monitoring of successful malaria control and eradication programmes in the Kumasi metropolis of Ghana.

MATERIALS AND METHODS

Mosquito larvae were collected from five different communities, Tafo Anyano,

Kwadaso, Ahodwo, Ayeduase and Abuakwa all within the Kumasi metropolis. All the communities lie approximately between (5°55´ and 7º10´N) and (1º25´ and 2º12´ W) and cover an area of about 8000 km^2 (Fig 3). The area falls within the equatorial climate zone (Walker, 1962) with a rainfall regime which is typical of the moist semi-deciduous forest zone of the country. The mean annual rainfall ranges between 1457 and 1488 mm. The mean monthly temperature is between 26 and 30 ^{0}C and the relative humidity ranges between 62 and 78 % (15).

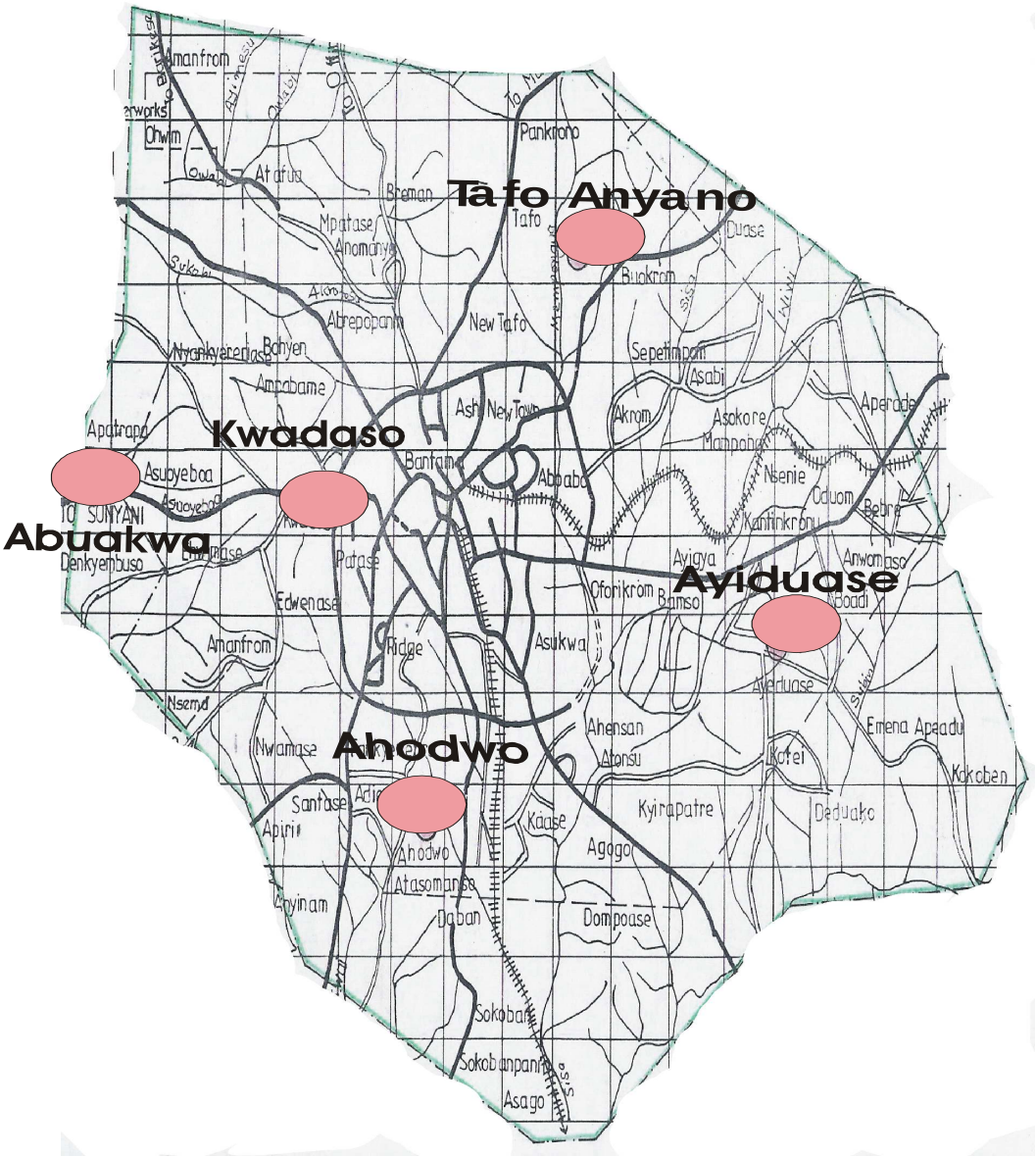

FIG. 1 Map showing the five communities within the Kumasi metropolis from which mosquito larvae were collected

Sample collection and identification of the *Anopheles* larvae

Mosquito larvae were collected, using a laddle, from diverse habitats including fresh shallow pools of water, construction and sand winning sites, gutters, vegetable farming sites and slow running streams. Most of the habitat had floating vegetation.

The larvae were transported to the laboratory in wide-mouthed labelled containers which were loosely covered to prevent suffocation, and under suitable condition. The larvae were transferred into white labelled plastic trays. *Anopheles* larvae were identified by their characteristic horizontal position below the surface of the water. The larvae were gently picked using a 3 ml rubber Pasteur pipette into a labelled 2.0ml eppendorf tubes containing absolute ethanol for storage prior to the DNA extraction.

DNA extraction

The DNA extraction method of (25), slightly modified, was used. Each mosquito larva was placed in a 1.5 ml Eppendorf tube in 100 ul Bender buffer (Appendix II) (pre- heated to 65 °C) and homogenized using a sterile polypropylene rod. This was followed by incubation at 65 ºC for 30 minutes and then the addition of 15 µl of pre-chilled 8M potassium acetate to each tube and mixed well by tapping the tube and each tube was then incubated on ice for 30 minutes, centrifuged at 14000 rpm for 10 minutes and supernatants transferred separately into fresh tubes. A volume of 250 µl of pre-chilled absolute ethanol was added to each supernatant and mixed well by inverting the tubes several times to precipitate the DNA. Each tube was incubated at -40°C for two hours followed by centrifugation at 14000 rpm to pellet the DNA and the supernatants discarded. The DNA pellets were washed with (200µl) 70% ethanol by centrifugation at 10000 rpm for 5 minutes. The supernatants were discarded and the tubes inverted over a paper towel and left to dry by evaporation. The dried DNA pellets were re-dissolved in 25 µl Tris EDTA + RNAse and then stored in the -20ºC freezer until ready for use.

Polymerase chain reactions

Molecular identification of *Anopheles gambiae*

The PCR method of (16) with species-specific oligonucleotide primers was used for the identification of the *An.gambiae*. The amplification process for the purpose of this work utilized one universal primer and three species-specific primers.

The sequence details of the primers that were abbreviated UN, GA, ME and AR with corresponding band sizes of the PCR products after electrophoresis on agarose gel is shown in Table 1. The universal primer UN annealed to the same position on the rDNA of each of the species of the *An. gambiae* complex except *An. Quandriannulatus* whose primer was not included. The GA annealed specifically to *An. gambiae s.l* ME to both *An. merus* and *melas*, and AR to *arabiensis*.

Table 1: *An. gambiae* s.l primer sequences, melting temperature and expected band sizes of the PCR amplified DNA products (16)

Primer	sequences (5'-3')	Tm (°C)	Band size (bp)
UN	GTGTGCCCCTTCCTCGATGT	56	468
GA	CTGGTTTGGTCGGCACGTTT	62	390
ME	TGACCAACCCACTCCCTTGA	90	464s
AR	AAGTGTCCTTCTCCATCCTA	78	315

PCR Amplification

The contents of the PCR reaction mix was thoroughly mixed centrifuged briefly at 10000 rpm and overlaid with oil to avoid evaporation and refluxing during thermo cycling. The amplification was carried out using a 100 PTC thermal cycler (MJ Research Inc., USA). The temperature profile for the reaction was 94 ºC for 3 minutes (initial denaturation) followed by 35 cycles of 94 ºC for 30 seconds (denaturation), 50 ºC for 30 seconds (annealing), 7 ºC for 1 minute (extension) and final cycle of 72 ^{0}C for 10 minutes followed by 4^{o}C for cooling. For each set of reactions a control which contained no DNA template was included.

Table 2: PCR reaction mix for *An. gambiae* s. l. species identification

Reagent	Volume(µl)	Final concentration
Sterile water	20.28	
10xReaction buffer	2.5	1x
20mM dNTPs	0.4	0.2mM
Primers: 10µMGA	0.3	0.3µM
10µMME	0.3	0.3µM
10µMAR	0.3	0.3µM
10µMUN	0.3	0.3µM
Taq(5U/µl) polymerase	0.13	0.5U
DNA template	0.5	
Final volume	25.0	

Analyses of PCR products

Gel electrophoresis was done with 2% agarose gel containing 0.5 mg/ml of ethidium bromide. After hardening of the gel, the comb was removed and the gel placed in an electrophoretic tank. 1X TAE buffer was poured into the tank till the gel was totally submerged. A volume of 8 µl PCR product was mixed with 1µl of 10X Bromophenol blue loading dye on a strip of Para film. Using either a mini or maxi-gel system the gels were run at 100V for 20 minutes. The bands were then visualized on an ultra violet trans-illuminator (UVP Dual-intensity Trans-illuminator, Upland, CA, U.S.A). A Polaroid direct screen instant camera fitted with an orange filter, a hood and a Polaroid type 667 film was used to take photograph of the bands. The film was processed as recommended by the manufacturers (Polaroid Inc., USA). The sizes of the PCR products were estimated by comparing with the mobility of a standard 100 bp DNA size marker (Sigma, USA).

Detection of knock down resistance (kdr) alleles in *Anopheles gambiae.*

The (16) method was used to detect kdr genes in the mosquitoes. A total of 50 samples, (10 for each site) positive for *Anopheles gambiae* s.s. by PCR were selected for the kdr detection. The kdr primers used were Agd_1, Agd_2, Agd_3 and Agd_4. The detail of the primer sequence is indicated in table 3. The contents and concentrations of the PCR reaction mix was thoroughly mixed centrifuged briefly at 1000 rpm and over laid with oil to avoid evaporation and refluxing during thermo cycling. Polymerase chain reaction products were analyzed as described under PCR analysis.

Table 3: Details of kdr primer sequences and their respective temperatures (17)

Primer	sequence 5' – 3'	Tm (^{0}C)
Agd1	ATAGAT TCC CCG ACC ATG	54
Agd2	AGA CAA GGA TGA TGA ACC	64
Agd3	AAT TTG CAT TAC TTA CGACA	40
Agd4	CTG TAG TGA TAG GAA ATTTA	52

Table 4: Kdr-PCR reaction mix for detection of the Kdr gene

Reagent	Volume(µl)	Final concentration
Sterile water	15.48	
10x Reaction buffer	2.50	1X
20mM dNTPs	0.40	0.2mM
Primers 10µM Agd_1	0.25	0.25µM
10µM Agd_2	0.25	0.25µM
10µMAgd_3	0.25	0.25µM
10µM Agd_4	0.25	0.25µM
Taq (5U/µl) polymerase	0.13	0.5U
DNA template	0.50	
Final volume	20.0	

RESULTS

PCR identification of siblings' species of *An. gambiae* s.l. of the larvae.

A total of 100 mosquitoes larvae were used for the PCR after their genomic DNA have been extracted. These comprised 20 samples from each of the five different sites used in this study. Out of the 100 larvae used, PCR amplification for species identification was successful for 76% while the other 24% were not identified. The 76 specimen were identified as *An.gambiae* s.s. as revealed by the size of 390 base pair fragment (Fig. 2).

Fig. 2. The percentage of PCR positive species identified as *An. gambiae* for all the sampling site

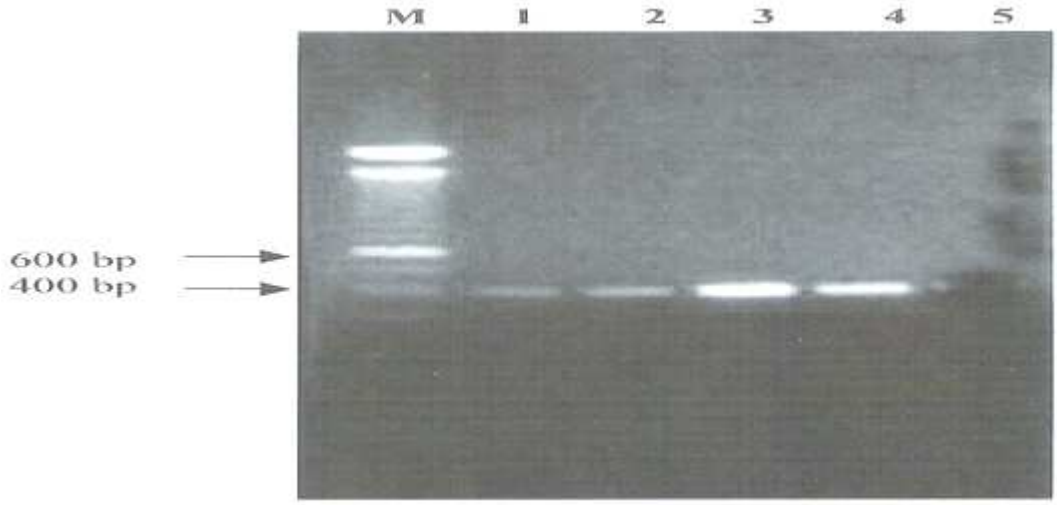

Figure 3: An example of 2.0% agarose gel electrophoresis of PCR amplified rDNA sequences of *An gambiae* ss larvae
Lane M = DNA molecular weight marker, lane 1-4 An. gambiae larvae, lane 5 = negative control.

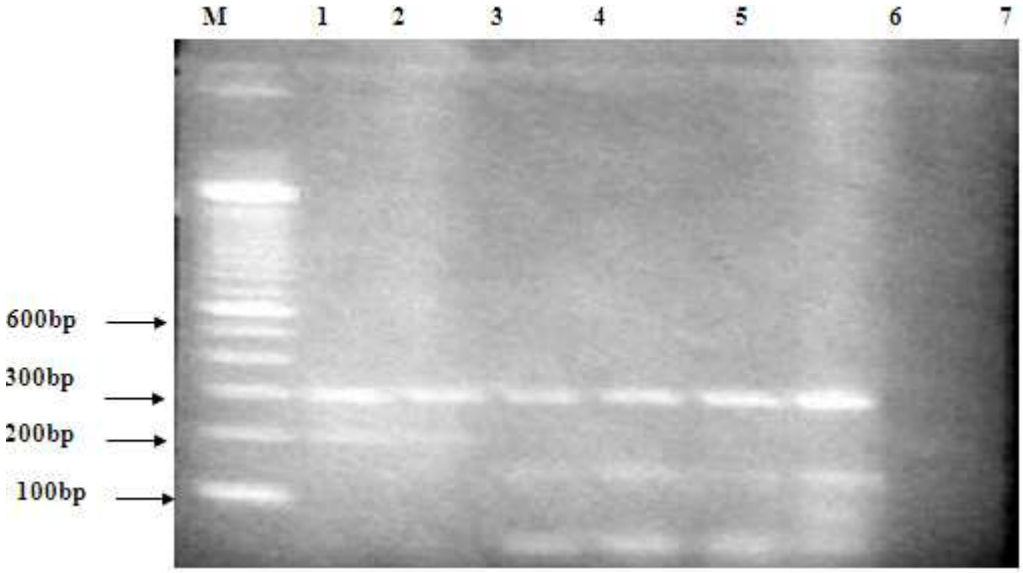

Table.5. Kdr status of *An. gambiae* s.s.

Sample Sites	Resistance (%)	Susceptible (%)
Ahodwo	(6) 60	(4) 40
Tafo Anyano	(8) 80	(2) 20
Abuakwa	(5) 50	(5) 50
Kwadaso	(7) 70	(3) 30
Ayiduase	(1) 10	(9) 90

N = 10
Where, N is the number of specimen per site

PCR determination of kdr mutation in the *An. gambiae*
A total of 50 PCR positive *An. gambiae* s.s. specimen (10 from each site) were selected for detection of the kdr status. Out of the 50, 27 (54%) were identified having the kdr gene whilst 23 (46%) were susceptible. An amplification product of 195 bp was revealed as positive for Kdr (resistant strain of *An. gambiae* s.s.) and 137 bp product also identified as negative (susceptible strain of *An. gambiae* s.s) with an internal control of 293 bp, as shown in the electrophoregram in figure: 3.

DISCUSSION

Polymerase chain reaction was used to determine the distribution of *Anopheles gambiae* species in the selected communities within the Kumasi metropolis. Only *An. gambiae s.s* was identified at all the sites. This result is consistent with previous work which reported by some researchers identified *An. gambiae* s.s as the only species in Greater Accra region which has similar climatic and environmental conditions as those of the sampling sites in present study sites in Kumasi ((18,19).

A total of 76 out of 100 samples were identified as *An. gambiae* s.s. by PCR (Fig.2). The other 24 specimens were not identified due the lack of specific primers for other sibling species. Again the lack of bands on gel electrophoresis could be possibly due to the DNA degradation prior to storage. However all the five sites have relatively high percentages of *An. gambiae* s.s. ranging between 70 and 90% (14 to 18 out of 20 samples for each site). The high numbers of the *Anopheles gambiae* s.s. which were identified in all the sites could be due to the species association with rain-dependent temporary sites than with permanent water bodies. These temporary fresh water collection sites were common to all the sampling sites. Again the annual relative humidity is between 62 and 78 % which is high and thus support the growth of *An. gambiae s.s.* population. Also the *An. gambiae* s.s. is associated with the human habitat (anthropophilic) (20) and since Kumasi is urban, this therefore could have been due to their high numbers.

Fifty four percent (27 out of 50 PCR positive for *An. gambiae,.s.s.*) possessed the kdr allele. Except Ayiduasi, which had the lowest value of 10% (1 out 10 samples) the other sites have relatively high percentages (50-80%) of kdr mutation gene (Table 5) which confers on them the resistance to insecticides. The widely accepted reason for this resistance in the West Africa populations of *An. gambiae* is the indiscriminate use of pyrethroid for agricultural purposes and for the control of household pests (20; 21, 22). The latter may be the case in these communities where there is high rate of urbanization and its consequent use of insecticides.

In some field population of a strain of *An. gambiae* studied in West Africa, resistance to a leucine –phenylalanine mutation was found (17) , while a leucine-serine mutations in the sodium channel was also found to confer resistance to Dichlorodiphenyltrichloroethane (DDT) and permethrin in a strain of *An gambiae* in Kenya, East Africa (23). The increased of indiscriminate use of these insecticides led to the ban of DDT in many countries in the 1970s due to the evolution of resistant mosquitoes in many regions. However the WHO currently advises the use of DDT to combat malaria in endemic areas (10) but with acceptable amount permitted under the Stockholm Convention on persistent organic pollutants. It has therefore recommended series of alternative insecticides to combat malaria in areas where mosquitoes are DDT-resistant, and slow the evolution of resistance among mosquitoes.

CONCLUSION

Anopheles gambiae s.s was identified (76%) using the available diagnostic oligonucleotide primers. Fifty four percent (27 out of 50 PCR positive samples) possess the kdr allele which confers on them the resistance to insecticides. The *An. gambiae* is also the major malaria vector. It is therefore necessary to sensitise public health institutions, legislative and other stake holders to educate the public about the need to improve upon environmental living conditions or sanitation in order to minimise thereby of *An. gambiae* s.s. This will prevent the rising malaria episodes in urban centres in the Kumasi metropolis of Ghana.

REFERENCES

[1] Breman J. (**2001**). *Am J, Trop Med Hyg* 64 (1, 2) S: 1-11.
[2] WHO/UNICEF (**2003**). The Africa malaria Report WHO/CDS/MAL/2003.1093. Retrieved 27th April 2003 from http://mosquito..who.int/amd2003/
[3] World Health Organization. (**1996**). Investing in health research for development. Report of the Ad Hoc Committee on Health Research Relating to Future Intervention Options; 1996 Report No. TDR/Gen/96.1. Geneva: The organization
[4] Greenwood, B. M., Bojang, K., Whitty, C. J., Targett, G. A. (**2005**) *Malaria Lancet* 365: 1487-1498.
[5] Aultman, K. S., Gottlieb, M., Giovanni, M. Y., Fauci, A. S. (**2002**). *Science*, 298:13.
[6] Centre for Health Statistics (**2010**). Ministry of Health, Ghana.
[7] World Health Organization (**2007**). World malaria report, Geneva.
[8] Centre for Health Statistics (**2009**). Ministry of Health, Ghana.

[9] World Health Organization. (**1999**). Test procedures for insecticide resistance Monitoring. in malaria vector, bio-efficacy and persistence of insecticides on treated surfaces. WHO, Geneva. WHO/CDS/CPC/MAL/98.12 unpublished document
[10] Service, M. W. (**1980**). A guide to medical entomology. Macmillan Press Ltd., London pp. 22-52
[11] Coluzzi, M. (**1984**) *Bull. Wld. Hlth. Org*. 62:107-113.
[12] Gillies, M. T. & Coetzee, M. (**1987**) *Publications of the South Africa Institute for Medical Research*, 55: 143-152.
[13] Fontenille, D. and Lochouarn, L. (**1999**). *Parasitologia* 41:267-271.
[14] Walker, H. O. (**1962**). Weather and climate. In Agriculture and land-use in Ghana. (ed. J.B. Wills). London. Oxford Univ. press Ch.2 pp.7-50.
[15] Scott, J. A., Brogdon, W. G. and F.H. Collins. (**1993**). *Am. J. Trop. Med. Hyg*. 49: 520-529.
[16] Martinez-Torres, D., Chandre, F., Williams, M.S., Darriet, F., Berge, J. B., Devonshire, A. L., Guillet, P., Pasteur. N and D Pauron. (**1998**) *Insect. Mol. Biol.* 7:179-184
[17] Adasi, K., M. D. Wilson and D. A. Boakye. (**2000**). Studies on insecticides usage and pyrethroid rsistance in populations of *An. Gambiae* s.s (Diptera ;Culicidae) in the Greater Accra region . WHO-MIM conference on insecticide resistance, Harare,Zambabwe.
[18] Midega, J. T. (**2001**). Cytological and molecular characterization of populations of the *Anopheles gambiae* Giles complex in a coastal savanna zone of Ghana. An MPhil. Thesis submitted to the University of Ghana
[19] Robert, G. and F. H. Collins. (**1996**). Anopheline mosquitoes and the agents they transmit. In: The biology of disease vectors (Barry. J.Beaty and Williams. C. Marquardt, Eds) University Press of Colorado.
[20] Darriet, G. F. (**1997**) Presence et evolution de la resistance aux pyrethrinoides et au DDT chez deux population d' *Anopheles gambiae* d'Afrique de l'ouest. WHO mimeographed document WHO/CTD/VBC/97.1001.
[21] Curtis, C. F., Miller, J .E., Hodjati, M. F., Kolaczinski, J. H and Kasumba, I. (**1998**) *Bull. Biol. Sci.* 353 (1376): 1769-75.
[22] Chandre, F., Darrier, F., Manga, L., Akogdeto, M., Faye, O., Mouchet, J. and Guillet, P. (**1999**) Status of pyrethoid effects associated with kdr mutation in *Anopheles gambiae*. Sensu lato. *Bull. WHO*. 77:230-234.
[23] Ransom, H., Jensen, B., Vulule, J.M., Wang, X., Hemingway, J and F.H. Collins. (**2000**) *Insect Mol. Biol*. 2:116-124.
[24] Collins, F. H., Mendez, M, A., Razmussen, M.O., Mehaffey, P. C., Besansky, N. J. and V. Finnerty. (**1987**) *Amer. J. Trop. Med. Hyg.* 37:37-41