East African Medical Journal Vol. 79 No 9 September 2002
MOLECULAR MARKERS IN EPIDEMIOLOGICAL MONITORING OF THE SPREAD OF RESISTANCE TO ANTIMALARIALS: A REVIEW
C.I. Nyamwange, BSc, MSc, Assistant Lecturer, and H. Nyamogoba, DN., BSc, MSc. Assistant Lecturer, Department of Medical Microbiology and Parasitology, Faculty of Health Sciences, Moi University, P.O. Box 4606, Eldoret, Kenya

Request for reprints to: Mr. C.I. Nyamwange, Department of Medical Biochemistry, Faculty of Health Sciences, Moi University, P.O. Box 4606 Eldoret, Kenya

MOLECULAR MARKERS IN EPIDEMIOLOGICAL MONITORING OF THE SPREAD OF RESISTANCE TO ANTIMALARIALS: A REVIEW

C.I. NYAMWANGE and H. NYAMOGOBA

ABSTRACT

Objective: **To review the prevalence and distribution of resistance to chloroquine and pyrimethamine-sulphadoxine combination and the use of molecular markers for monitoring the spread of the resistance.**
Data sources: **Literature search on compact disk-read only memory (CD-ROM), Medline and Internet, using the key words: Malaria and epidemiology, malaria and resistance, sulphadoxine-pyrimethamine resistance and chloroquine resistance. Some articles were manually reviewed.**
Study selection: **Relevant studies or articles on resistance to chloroquine, sulphadoxine pyrimethamine combination and other antimalarials and molecular resistance markers from various sources are included in the review.**
Data extraction**: From individual study or articles.**
Data synthesis: **Information on antimalarial resistance is harmonised under the headings; Introduction, Prevalence and distribution of resistance to antimalarials, Use of molecular markers for epidemiological monitoring of antimalarial resistance.**
Conclusion: **The spread and status of resistance to sulphadoxine-pyrimethamine (SP) and chloroquine should be monitored constantly in major health facilities. This will not only detect the emergence of resistance to these drugs but also generate information on the extent of resistance to these antimalarials. Mutations in the *dhfr* and *dhps* genes can be used as markers in SP resistance surveilance while the presence of pfcrt mutations thought to confer resistance should also be analysed to ascertain whether they truly correlate to the resistance patterns that have been observed in various malarious regions. Little is known on the interaction and exact role(s) of PfCRT protein in conferring the resistance trait.**

INTRODUCTION

Each year there are more than 500 million malaria infections worldwide, which lead to at least 2 million deaths. In Sub-Saharan Africa alone, at least 1 million people die of malaria each year, majority being children under five(1). Asia, South and Central America are also home to some of the worst malaria episodes.

Chloroquine (CQ) has been the most important antimalarial for more than 40 years(2). However, its value has been severely compromised by the emergence of chloroquine resistant *Plasmodium falciparum* strains, which were initially reported from South America(3) and South-east Asia(4). Since then, chloroquine resistance has spread to most parts of the malaria zones including sub-Saharan Africa. Resistance to antimalarials is mainly of practical imporance in relation to *P. falciparum* strains, which are not only resistant to chloroquine but also to amodiaquine and the pyrimethamine-sulphadoxine combination(5).

Studies on genetic diversity of many genes show that there is considerable allelic diversity of many genes particularly those encoding for antigens, enzymes and proteins(6) and, in the sequence of many genes(7,8). No two isolates have been found in which parasites possess identical genotypes even in a small community in which malaria transmission is highly seasonal. Mixed infections involving more than one clone are common, with geographical variation in the frequencies in which alleles of many genes occur(9).

Physical mapping by Su *et al.*(10) has revealed that chloroquine resistance domain in *P. falciparum* maps as a mendelian trait to a 36kb segment of chromosome(7). This segment harbours *P. falciparum* CQ-resistance transporter (*pfcrt*) gene that encodes a unique protein with complex polymorphisms. The PfCRT protein is found at the periphery of the digestive vacuole, where chloroquine exerts its activity by inhibiting haeme detoxification.

Fidock *et al*(8) have reported unique (*pfcrt*) mutations located in the chloroquine resistant phenotype that are strongly associated with chloroquine resistance. It is important for malariologists to assess the polymorphisms of this gene in isolates from areas where resistance to

CQ has been observed vis-a-vis their role in chloroquine efflux from the food vacuole of resistant strains.

The spread of chloroquine resistance in Africa has led to the use of pyrimethamine and proguanil, and their combinations with sulphonamides as alternative first line drugs for the treatment and prevention of *falciparum* malaria(11). Drug-resistant parasites have been selected rapidly in areas where SP combination has been used(12), raising lots of concern about its dependability as the first drug of choice for chloroquine resistant infections.

Prevalence and distribution of resistance to antimalarials: There has been a worldwide spread of chloroquine resistance since it was first reported in Thailand and Colombia in 1959(13). According to Clayde(14), chloroquine resistance emerged in the Eastern Africa region in 1978 and its epicentre was in East Africa. Chloroquine resistant strains are now found in Southeast Asia, the Amazon region of South America and sub-Saharan Africa(15). Heavy and improper use of drugs may contribute to selection of resistant strains of malaria parasites.

The status of *P. falciparum* resistance to chloroquine in Sabah, Malaysia were not known until 1971-1975 when *in-vivo* and *in-vitro* studies showed that 51% of the 57 cases studied were resistant to chloroquine. By 1975, chloroquine resistance was a well established entity in whole of South East Asia, and still presents a problem of increasing importance. In 1979 there was a radical change of treatment for *P. falciparum* infection from chloroquine to SP (fansidar) in Sabah State(16). In Papua New Guinea, al-Yamal *et al.* (17) have reported that 85% of the infections have some degree of resistance to chloroquine of which 52% were at RI level (parasites disappear from the peripheral blood following standard chloroquine therapy but recrudescence within 28 days), 26% at RII level (parasitaemia is reduced by over 75% during chloroquine therapy but parasites persist in the peripheral circulation) and 22% at RIII level (parasitaemia is reduced by less than 75% or is unaffected by standard doses of chloroquine), 86% of the infections were resistant to amodiaquine while 7% were resistant to quinine. High prevalence of mefloquine resistant *P. falciparum* has been reported in Thailand(18), and of halofantrine and amodiaquine in Kenya(19).

The existence of chloroquine resistant *falciparum*(20) and *vivax* malaria(21) have been confirmed in Bombay, India. However, it is worth noting that chloroquine resistance has not been a problem with the other *Plasmodium* species in all the malarious zones.

The first case of chloroquine resistant *falciparum* malaria in Rwanda was reported in 1981(22). Since 1983, several cases of chloroquine resistant malaria have been observed in Caucasian adults living in the Central African Republic (CAR) despite an antimalarial prophylaxis. Between 1984 and 1985, several *in vitro* and *in vivo* studies in CAR by Delmont *et al*(23) in cohorts of children to determine sensitivity of *P. falciparum* to chloroquine found that therapeutic response to amino-4-quinolines was satisfactory as compared to the neighbouring countries. In Brazzaville, Congo, Carme *el al*(24) have assessed the resistance to chloroquine and amodiaquine of *P. falciparum*. They have reported resistance of 38.7% (9.7% at the RII level, 29% at the RI level) to chloroquine and 21.2% (3% at the RII levels, 18.2% at the RI level) to amodiaquine. Based on these results, they have concluded that there is emergence and rapid spread of resistance to these two drugs. Other cases of chloroquine resistant *falciparum* malaria among travellers from Central and West Africa to South Africa were reported in 1981(25). In Zimbabwe, a study by Mahomva *et al*(26) has shown a high level of resistance of 33% of the study population to chloroquine, at RII and RIII levels.

Alene *et al.*(27) have shown a 4% chloroquine resistance within the study population in Central Ethiopia. The resistance was originally thought to be confined to border areas but this study has shown it to be highest in the central region, particularly in the lowlands.

The sensitivity of *P. falciparum* to chloroquine has been tested in Muheza, Pangani, Tanga and Korogwe districts in northeastern Tanzania by both *in vivo* and *in vitro* tests in school children. A total dose of 25 mg/kg body-weight given over a period of three days failed to clear asexual parasites from peripheral blood by day seven in 12.5% of the children tested at Muheza, 5.9% at Pangani, 31.8% at Tanga, and 39.5% at Korogwe(28).

There are reports of continuing increase of chloroquine resistant infections in Kenya. Resistance to the 25 mg/kg standard dose of chloroquine was first reported among tourists visiting the Mount Kilimanjaro region in 1978(29). Since then, the resistance has spread centrifugally with increasing rapidity, partly as a result of the increased population mobility particularly by asymptomatic carriers of resistant infections. However, studies by Oloo *et al.*(30) did not show evidence of chloroquine resistance in some parts of the lake basin of Kenya by 1986. This contrasted with continuing reports of proven chloroquine-resistant *falciparum* malaria in non-immune visitors who acquired infections in Kenya(30). Studies in Kilifi, Kenya, suggest that failure to clear the parasites in 45.9% of multigravidae patients is due to the presence of chloroquine resistant parasites, predominantly at the RI, RII levels and fewer at RIII level(31).

In Bondo district of Nyanza Province, Njagi *et al*(32) have studied the effectiveness of chloroquine and sulphadoxine/pyrimethamine (SP) combination. They have found that fourteen days after treatment, chloroquine showed a clearance rate of 42.3% whereas the SP combination had a clearance rate of 95.7%. In their study during a highland malaria epidemic in Uasin Gishu district, Kenya, Khan *et al.*(33) have reported resistance to chloroquine in 11 % of the patients fourteen days after treatment with chloroquine.

Drug-resistant parasites have been selected rapidly in areas where SP combination has been used(16). Extensive pyrimethamine-sulfadoxine (Fansidar) resistance was first demonstrated in eastern Thailand in 1980(34). The frequency of resistance to this combination has since increased, such that in Southeast Asia and the Amazon, the combination has been found ineffective against *falciparum* malaria. The combination is still useful in most of tropical Africa(15,35). However, between 42% and 63% pyrimethamine resistance has been reported in Cameroon(36).

The spread of chloroquine resistance in Africa has lead to the use of the dihydrofolate reductase (DHFR) inhibitors pyrimethamine and proguanil, and their combinations with sulphonamides as alternative first line drugs for the treatment and prevention of falciparum malaria(11). For example, in the early 1990s, sulphadoxine-pyrimethamine was adapted in Malawi as the drug of choice for chloroquine resistant *falciparum* malaria, but resistance to this combination was reported within three years of its use(11). Also resistance to the pyrimethamine and proguanil has been reported in Tanzania where it is often used for prophylaxis against malaria(37), Senegal, Kenya and Niger(38).

A study of SP effectiveness carried out between 1997 and 1999 in Kilifi, at the Kenyan coast by Mberu *et al.*(39) has shown the emergence of resistance to sulphadoxine-pyrimethamine at RI and RII levels. A follow up study by Nzila *et al.*(12) has associated triply mutated allele of DHFR alone or with mutant DHPS alleles to RI and RII resistance levels.

Nzila *et al.*(40) have compared the selective pressure exerted by the slowly eliminated Sulphadoxine-pyrimethamine (SP) and the more rapidly eliminated combination of chlorproguanil-dapsone by analysing for point mutations in dihydrofolate reductase and dihydropteoralte synthase genes. They have found that most of the patients treated with SP return with recrudescent infections. Consequently, they have suggested that chlorproguanil-dapsone combination is a preferable alternative for treatment of chloroquine resistant *falciparum* malaria in sub-Saharan Africa. This is in agreement with reports by Mutabingwa(49) indicating a 7-40% increase in resistance to SP within three years of its adoption in 1997 as first-line treatment of malaria in Kenya.

Use of molecular markers for epidemiological monitoring of drug resistance: Molecular markers for drug resistance can be used to screen for antimalarial resistant infections in large populations in malaria endemic areas. The ability to use molecular markers to monitor resistance against antimalarials in field studies will enable scientists to rationalise recommendations for changing the treatment policy in time(11). Molecular epidemiological studies on drug resistance might provide further insight into the molecular mechanisms underlying its development. Only molecular-epidemiological monitoring of parasite populations can confirm the true association of molecular mechanisms identified in the laboratory with drug resistance in the field. This is an important issue with respect to the molecular mechanisms involved in chloroquine and sulphadoxine-pyrimethamine resistance.

Fidock *et al.*(8) have shown that all old and new world pfcrt alleles in the CQR parasites consistently include mutations for K76T and A220S. These observations are consistent with findings that CQR parasites from all regions have comparable verapamil-reversible phenotypes. These findings have been reinforced in a study conducted by Djimde *et al.*(42) in Mali which has demonstrated the presence of K76T to T761 mutation in the *pfcrt* gene in all post-chloroquine treatment infections. This shows that parasites carrying the T761 mutation are most likely to be resistant to chloroquine. This mutation can be used as a marker in the surveillance of chloroquine resistant *falciparum* malaria.

Resistance to sulphadoxine-pyrimethamine (SP) combination commonly used in treatment of chloroquine resistant malaria is due to a key point mutation in the dihydrofolate reductase-thymidylate synthase genes of *P. falciparum,* which results in the substitution of the wild type alleles er-108 by either Asn-108 or Thr-108(34). Mutation specific PCR and allelic-specific restriction analysis (ASRA) of codon *108* is a good indicator of the absence or presence of resistance.

Several polymorphisms have been identified in the *P falciparum dhps* gene, which may correlate with sulphadoxine-resistance. Simple and rapid tests have been developed to detect these polymorphisms, using PCR followed by restriction digestion(43). These tests can accurately identify all the polymorphisms described to date at codons, 540, 581, 613, 436 and 437 in the *dhps* gene and, at codons 16, 51, 59, *108,* and 164 in the *dhfr* gene. These assays are invaluable in evaluating the contribution of specific base changes in the *dhps* and *dhfr* genes of the parasite to the sulphadoxine resistance phenotype and to the clinical failure of the sulphadoxine-pyrimethamine combination(44). Studies by Plowe *et al.*(53) have shown the prevalence of mutations at DHFR codon 108 and a new mutation at DHPS 540 to correlate with increased pyrimethamine-sulphadoxine resistance. Mutations at DHFR 164 and DHPS 581 are common in Bolivia, where pyrimethamine-sulphadoxine resistance is widespread, but absent in African sites(44). They are of the opinion that DHFR and DHPS mutations occur in a progressive and stepwise fashion. They recommend the identification of specific sets of mutations that cause *in vivo* drug failure, which may lead to the development of molecular surveillance methods for pyrimethamine-sulfadoxine resistance.

The East African Network for Monitoring Antimalarial Treatment (EANMAT) has been monitoring the sensitivity of malaria parasites to SP once every year at four sentinel sites in each of the target countries (Kenya, Tanzania, Uganda and Malawi) since 1998. The network has established that resistance at these sites is past the alert phase of 6-14%.

CONCLUSIONS

Malaria is a major scourge afflicting millions of people every year, which is further complicated by resistance to common antimalarials such as chloroquine and pyrimethamine-sulfadoxine. The development of resistance to antimalarial agents has spread rapidly since the 1950s, culminating in the widespread distribution of multiple drug-resistant strains of *P. falciparum* in most endemic areas.

In Southeast Asia SPs are no longer effective and treatment is changing to newer drugs such as mefloquine, halofantrine and artemisinin. There is a danger that *P. falciparum* parasites may rapidly develop resistance even to these new compounds unless the use of such compounds is carefully controlled. Self-medication, which is widely practiced and usually involves under doses may contribute towards the development of resistance to antimalarials due to selection of resistant strains of malaria parasites.

There is limited capacity to monitor resistance at the molecular level in most developing countries due to lack or limited budgetary allocation, shortage of skilled personnel and poor dissemination of available information and technology.

Mutations in the *pfcrt* (CQR) gene, and *dhps* and *dhfr* genes are responsible for chloroquine and pyrimethamine-sulfadoxine resistance respectively. Whereas much is known about the *dhps* and *dhfr* genes products, little is understood about the pfCRT protein's interaction with and transport of chloroquine. Does this protein associate with other aminoquinolines? If so, how?

Chloroquine resistance is rampant in sub-Saharan Africa, which has lead most countries to change from chloroquine to pyrimethamine-sulphadoxine combination as the first-line drugs against malaria. The trouble is that these drugs were in circulation long before these changes were declared as policies to the extent that resistance to them might have already developed.

RECOMMENDATIONS

The studies on diversity of the molecular resistance markers with respect to various endemic states should be carried out in various regions as it may help determine the extent of the variation that occurs in these genes within small communities. It may also establish whether these changes occur over years, which in turn will guide the planning of chemotherapeutic and vaccination control measures.

There is need for further surveys using mutation T761 in *pfcrt* gene in monitoring and assessing the situation on chloroquine resistance and mutations in codons 108 and 540 of the *dhfr* and *dhps* genes respectievly as markers for the monitoring of the spread of SP resistance. The National Malaria Control Programme in collaboration with research institutions and universities ought to establish national and regional resistance monitoring centres in all major hospitals. These centres will be receiving samples from health centres within their catchment areas quarterly for analysis. Budgetary allocations towards antimalarial resistance surveillance and formulation of new combinations ought to be solicited and provided for in the national budgets.

Molecular epidemiological studies on drug resistance ought to be carried out so as to provide further insight into the molecular mechanisms underlying the development of resistance and to confirm the true association of molecular mechanisms identified in the laboratory with drug resistance in the field.

In as much as reliance should not be placed on drugs alone to control malaria in a community, it is vital that all measures possible should be taken to protect such new compounds by judiciously selecting combinations with other antimalarials and ensuring that essential procedure is followed in their dispensation. The possibility of using short acting antimalarials as opposed to long acting antimalarials need to be explored because the long acting drugs give the parasites chance to select for resistance. Further there is urgent need for accelerated research on reversing the existing resistance by use of resistance reversers, chloroquine enhancers and novel drug combinations.

REFERENCES

1. Myrvang, B. and Godal, T. WHO's malaria program Roll Back Malaria. *Tidsskrift for Den Norske Laegeforening.* 2000; **120:** 1661-1664.
2. Krogstad, D. J. Malaria: a re-emerging disease. *Epidemiology review.* 1996; **8:**77-89.
3. Moore, D.V., Lanier, J.E. Observations on two *plasmodium falciparum* infections with abnormal response to chloroquine, *Amer. J. Trop. Med. Hyg.* 1961; **10**:2-9.
4. Harinasuta, T., Migasen, S., Bunnag, D. UNESCO First Regional Symposium on Scientific Knowledge of Tropical Parasites. University of Singapore Nov 5-9, 1962. Singapore, Malaysia, University of Singapore. 1962. pp 143-153.
5. Sandosham, A.A., Fredericks, H.J., Ponnampalam, J.T., Seow, C.L. Ismail, O. Othman, A.M. and Ali, A.R. Chloroquine resistant malaria in West Malaysia. *J. Trop. Med. Hyg.* 1975; **78:**54-58.
6. Creasey, C.L., Fenton, B., Walker, A., Thaithong, S., Oliveira, S., Mutambu, S. and Walliker, D. Genetic diversity of *Plasmodium falciparum* shows geographical variation. *Amer. J. Trop. Med. Hyg.* 1990; **42:**403-413.
7. Engelbretch, F., Togel, E., Beck, H.P. Enwezor, F., Oettli, A. and Felger, I. Analysis of *Plasmodium falciparum* infections in a village community in Northern Nigeria: determination of msp2 genotypes and parasite-specific IgG responses. *Acta Tropica.* 2000; **74:**63-71.
8. Fidock, D.A., Nomura, T., Talley, A. K., Cooper, R.A., Dzekunov, S. M., Ferdig, M.T., Ursos, L.M.B., Sidhu, S.A., Naude, B, Deistsch, K.W., Su, X., Wootton, J.C., Roepe P.D. and Wellems, T.E. Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. *Molecular Cell.* 2000; **6:**861-871.
9. Babiker, H.A., Creasey, A.M., Fenton, B., Bayoumi, R.A. Arnot, D.E. and Walliker, D. Genetic diversity of *Plasmodium falciparum* in a village in Eastern Sudan.1. Diversity of enzymes, 2D-PAGE proteins and antigens. *Trans. Roy. Soc. Trop. Med. Hyg.* 1991; **85**:572-577.
10. Su, X., Kirkman, L.A., Fujioka, H. and Wellems T.E.

Complex polymorphisms in' an approximately 330-kDa protein linked to chloroquine resistance *P. falciparum* in Southeast Asia and Africa. *Cell.* 1997; **91:**593-603.

11. Plowe, C.V. and Wellems, T.E. Molecular approaches to the spreading problem of drug resistant malaria. *Adv Exp. Med. Biol* 1995; **390:**197-209.
12. Nzila, A.M., Mberu, E.K. Sulo, J. Dayo, H., Winstanley, P.A., Sibley, C.H. and Watkins, W.M. Towards an understanding of the mechanism of pyrimethamine-sulfadoxine resistance in *Plasmodium falciparum* genotyping of dihydrofolate reductase and dihydropteroate synthase of Kenyan parasites. *Antimicrobial Agents and Chemotherapy.* 2000; **44:** 991-996.
13. Chwatt, B. Chemotherapy of malaria, 2nd edn. Geneva. World Health Organization. 1986.
14. Clayde, D. F. Variations in response of malaria parasites to drugs in Asia and Oceania. *La Medicina Tropicalle Vela Cooperazione allo Sviluppo.* 1987; **3:**3-22.
15. Kager, P.A. and Wetsteyn, J.C. Malaria and drug resistance. *Nederlands Tijdshrift voor Genee.* 1996; **140:** 151-155.
16. Maqsudur Rahman K.M. Study of *Plasmodium falciparum* resistance to 4-Aminoquinolines (Chloroquine) in Sabah. *Malaysian J. Trop. Med. and Hygiene.* 1980; **83:**259-264.
17. al-Yaman, F, Genton, B., Mokela, D., Narara, A., Raiko, A. and Alpers, M.P. Resistance of *Plasmodium falciparum,* malaria to amodiaquine, chloroquine and quinine in the Madang Province of Papua New Guinea 1990-1993. *Papua New Guinea Med. J.* 1996; **39:** 16-22.
18. Plasai, V. and Spielman, A. Mefloquine insusceptibility of malaria in Thailand not promoted by non-regulated drug use. *Acta. Tropica.* 1996; **60:** 281-289.
19. Anabwani, G.M., Esamai, F. O. and Menya, D.A. A randomised controlled trial to assess the relative efficacy of chloroquine, amodiaquine, halofantrine and fansidar in treatment of uncomplicated malaria in children. *East Afr. Med. J.* 1996; **73:**155-158.
20. Garg, M.R., Gogtay, N.J. and Kshirsagar, N.A. Efficacy of a sulfadoxine/pyrimethamine in chloroquine resistant falciparum malaria in Bombay. *J Ass Phys. India.* 1996; **44:** 683-685.
21. Potkar, C.N., Kshirsagar, N.A. and Kathuria, R. Resurgence of malaria and drug resistance in *Plasmodium falciparum* and *Plasmodium vivax* species in Bombay. *J. Ass. Phys. India.* 1995; **43:** 336-338.
22. Mafart, Y., Pieron, R., Melchior, J.C. A new case of *Plasmodium falciparum* malaria resistance to chloroquine in Africa (Rwanda). *Medecine Tropicale.* 1981; **41:** 677-680.
23. Delmont, J., Testa, J., Monges, P., Limbassa, J. Georges, A.J. and Faugere, B. Status of drug resistance of *Plasmmodium falciparum* in the Central African Republic. Results of studies carried out between 1984 and 1986. *Bulletin de la Societe de Pathologie Exotique et de Ses Filiales.* 1987; **80:**434-442.
24. Carme, B., Mbitsi, A., Moudzeo, H., Ndinga, M., Eozenou, P. Drug resistance of *Plasmodium falciparum* in the Congo. 2. Comparative study *in vivo* of chloroquine and amodiaquine in Brazzaville schoolchildren (November 1986). *Bulletin de la Societe de Pathologie Exotique et de Ses Filiales.* 1987; **80:**426-433.
25. Mahoney, J.L. Malaria 'breakthroughs' and resistance to chloroquine in Africa. Case reports. *South Afr. Med. J.* 1981; **60:**786-788.
26. Mahomva, A. I., Petreson, D.E. and Rakata, L. Chloroquine resistant.*falciparum* in Mutare District, eastern Zimbabwe. *Central Afr. J. Med.* 1996; **42:**112-113.
27. Alene, G.D. and Bennett, S. Chloroquine resistance of *Plasmodium falciparum* malaria in Ethiopia and Eritrea. *Trop. Med. Int. Hlth.* 1996, **1:** 810-815.
28. Kilimali, V.A. and Mkufya, A.R. *In vivo* and *in vitro* assessment of the sensitivity of *Plasmodium falciparum* to chloroquine in four districts of Tanga region,Tanzania. *Trans. Roy. Soc. Trop. Med. Hyg.* 1985; **79:** 478-481.
29. Schapira, A. The resistance of falciparum malaria in Africa to 4-aminoquinolines and antifolates. *Scandinavian J. Infec. Diseases-Supplementum.* 1990; **75**:1-64.
30. Oloo, A.J., Kamunvi, F. and Owaga, M.L. Response of *falciparum* Malaria *in vivo* to standard regimen of chloroquine in Kisumu District, Western Kenya. *Trans. Roy. Soc. Trop. Med. Hyg.* 1986; **80:**532-533.
31. Rukaria, R.M., Ojwang, S.B.O., Oyieke, J.B. and Sekadde, K.C.B. *In vivo* and *in vitro* response of *Plasmodium falciparum* to chloroquine in pregnant women in Kilifi District, Kenya. *East Afr. Med. J.* 1992; **9:**306-310.
32. Njagi, J.K., Estambale, B.B.A., Magnussen, P., Ouma, J.H., Rapuoda, B. A. and Kabiru, E.W. Effectiveness of chloroquine and sulfadoxine/pyrimethamine combination in the treatment *falciparum* malaria in a malaria endemic area in Kenya. *East Afr. Med. J.* 1998; **75:**446-449.
33. Khan, B., Ofulla, V.O., Kariuki, D.M., Githure, J.I., Kabiru, E,W. and Martin, S.K. Drug sensitivity studies during highland malaria epidemic in Kenya. *Trans. Roy. Soc. Trop. Med. Hyg.* 1992; **86:**371-372.
34. Reacher, M., Campbell, C.C., Freeman, J., Doberstyn, E.B. and Brandling-Bennett, A,D. Drug therapy for *Plasmodium falciparum* malaria resistant to pyrimethamine-sulfadoxine (Fansidar). A study of alternate regimens in Eastern Thailand. *Lancet.* 1980; **8255**:1066-1069.
35. Watkins, W.M. Pharmacology and pharmacokinetics of new antimalarials. *Medicine Tropicale.* 1995; **55:** 33-36
36. Basco, L.K. and Ringwald, P. Molecular epidemiology of malaria in Younde, Cameroon IV. Evolution of pyrimethamine resistance between 1994 and 1998. *Amer. J. Trop. Med. Hyg.* 1999; **61:** 802-806.
37. Edoh, D., Mshinda, H., Jenkins, J. and Burger, M. Pyrimethamine-resistant *Plasmodium falciparum* parasites among Tanzanian children: a facility-based study using polymerase chain reaction. *Amer. J. Trop. Med. Hyg.* 1997; **57:** 342-347.
38. Parzy, D., Doerig, C., Pradines, B., Rico, A., Fusai, T. and Doury, L.C. Proguanil resistance in *Plasmodium falciparum* African isolates: assessment by mutation-specific polymerase chain reaction and *in vitro* susceptibility testing. *Amer. J. Trop. Med. Hyg.* 1997; **57:** 646-650.
39. Mberu, E.K., Mosobo, M.K., Nzila, A.M., Kokwaro, G.O., Sibley, C.H. and Watkins, W.M. The changing *in vitro* susceptibility pattern to pyrimethamine/sulfadoxine in *Plasmodium falciparum* field isolates from Kilifi, Kenya. *Amer. J. Trop. Med. Hyg.* 1997; **62:** 396-401.
40. Nzila, A.M., Nduati, E., Mberu, E.K., Hopkins, S.G., Monks, S.A, Winstanley, P. A. and Watkins, W.M. Molecular evidence of greater selective pressure for drug resistance by the long-acting antifolate pyrimethamine-sulfadoxine compared with the shorter-acting chlorproguanil/dapsone on Kenyan *Plasmodium falciparum. J. Infec. Dis.* 2000; **181:** 2023-2028.
41. Mutabingwa, T.K. Chairman's column. The newsletter of the East African network for monitoring antimalarial treatment. 2001; **4:** 1-2.
42. Djimde, A., Doumbo, O.K., Cortese, J.F. Kayentao, K., Doumbo, S., Diourte, Y., Dicko, A., Su, X., Nomura, T., Fidock, D.A., Wellems, T.E. and Plowe, C.V. A molecular Marker for chloroquine-resistant.*falciparum* Malaria. *New. Eng. J. Med.* 2001; **344:** 257-263.
43. Duraisingh, M.T., Curtis, J. and Warhurst, D.C. *Plasmodium falciparum*: detection of polymorphisms in the dihydrofolate reductase and dihydropteroate synthetase genes by PCR and restriction digestion. *Exp. Parasit.* 1998; **89:** 1-8.
44. Plowe, C.V., Cortese, J.F., Djimde, A., Nwanyanwu, O.C., Watkins, W.M., Winstaffley, P.A., Estrada-Franco, J.G., Mollinedo, R.E. Avila, J.C. Cespedes, J.L., Carter, D. and Doumbo, O.K. Mutations in *Plasmodium falciparum* dihydrofolate reductase and dihydropteroate synthase and epidemiologic patterns of pyrimethamine-sulfadoxine use and resistance. *J. Infectious Diseases.* 1997; **176:**1590-1596.