

35. Chirikov, V., *Phys. Rep.*, 1979, **52**, 263–379.
 36. Walker, L. H. and Ford, J., *Phys. Rev.*, 1969, **188**, 416–432.
 37. Grossmann, F., Dittrich, T., Jung, P. and Hänggi, P., *Phys. Rev. Lett.*, 1991, **67**, 516–519.
 38. McKay, R. S. and Meiss, J. D., *Phys. Rev. A*, 1988, **37**, 4702–4706; Hanson, J. D., Cary, J. R. and Meiss, J. D., *J. Stat. Phys.*, 1985, **39**, 327–345.

ACKNOWLEDGEMENTS. We thank CSIR, New Delhi for financial assistance and a referee for constructive criticism.

Received 18 September 2001; revised accepted 28 January 2002

Pyrethroid resistance in *Anopheles culicifacies* in Surat district, Gujarat, west India

O. P. Singh, K. Raghavendra, N. Nanda, P. K. Mittal and S. K. Subbarao*

Malaria Research Centre (ICMR), 22 Sham Nath Marg, Delhi 110 054, India

A focus of deltamethrin resistance in *Anopheles culicifacies*, the major vector of malaria in India, was identified in Surat district, Gujarat, western coast of India, where two synthetic pyrethroids, deltamethrin and cyfluthrin are being used under the public health programme since 1996, as a selective vector control measure. The per cent mortalities in *An. culicifacies* after one-hour exposure to 0.05% deltamethrin varied from 60 to 78 and LT_{50} and LT_{90} values were 27 to 38 and 164 to 218 min, respectively in different localities; whereas laboratory-maintained pyrethroid susceptible strains showed 100% mortality even at exposure for 10 min. The population also showed high knock-down resistance against 0.05% deltamethrin; the knock-down times, KDT_{50} and KDT_{90} , were 74–81 and 217–297 min respectively as against 8.8–10.7 and 14.2–15.7 min respectively in pyrethroid-susceptible, laboratory-colonized strains of *An. culicifacies* species B and C. The *An. culicifacies* population in the study area was found to comprise of two sibling species, B and C, which did not differ in knock-down susceptibility to deltamethrin.

ANOPHELES culicifacies is a principal vector of malaria in rural and peri-urban areas of India, which alone contributes 60–65% of new malaria cases each year¹. *An. culicifacies* complex is comprised of five sibling species provisionally designated as A, B, C, D and E^{2,3}. Distinct

biological variations exist between members of the complex, of which the most important from vector-control point of view are differences in their role in malaria transmission^{3–5}, susceptibility to malaria infection^{6,7}, and response to insecticides^{8–10}. The main strategy of control of *An. culicifacies* in rural areas is indoor spraying of residual insecticides. Spraying of DDT and HCH under the public health programme was introduced in the 1950s. A few years after the introduction of these insecticides, *An. culicifacies* developed resistance to DDT^{11,12}, dieldrin¹³ and BHC¹⁴. As a result, malathion was introduced in Gujarat and Maharashtra in 1969 against which *An. culicifacies* developed resistance rapidly by 1973 (ref. 15). Later this species was reported to be triple-resistant in Gujarat, Andhra Pradesh and Haryana^{9,10}. As a result, synthetic pyrethroids (SPs) were introduced in some parts of India in the 1990s for selective control of the multiple insecticide-resistant malaria vector/s in high-risk areas.

Gujarat is one of the highly malarious states in India, with Surat district responsible for the maximum number of malaria cases among all districts of the state. In Gujarat *An. culicifacies* has developed resistance to all insecticides used earlier, namely DDT, HCH and malathion⁹. SPs were therefore introduced in some areas of Gujarat with high malaria risk, including Surat district in 1996 as a selective malaria vector-control measure. The present study was undertaken in Surat district to monitor the status of pyrethroid resistance in *An. culicifacies*.

Surat district is situated on the western coast of India between 21–22°N latitude and 73–74°E longitude. Villages under three Primary Health Centres (PHC) of two talukas (sub-division of district) of Surat district which have different history of insecticide selection pressure, were selected for the study. The study area is predominantly hilly and forested. *An. culicifacies* is the predominant species among anophelines and is the main malaria vector in the area. The study villages and history of insecticides used in these villages since 1995 are given in Table 1. Under Amladam PHC, all 29 villages are under indoor residual spraying of cyfluthrin/deltamethrin since 1996, whereas in other PHCs, villages with higher malaria risk are covered with these insecticides.

To monitor the susceptibility status, indoor resting *An. culicifacies* females were collected from the study villages in the morning (6–8 a.m.) using an aspirator and torch light. Blood-fed mosquitoes were exposed to 0.05% deltamethrin-impregnated paper, a revised diagnostic dose recommended by WHO¹⁶, using standard WHO's adult susceptibility test kit for 5, 10, 15, 30, 60, 120 and 240 min, and were held for 24 h recovery period with access to cotton pad soaked with 5% glucose solution in water. Mortality was recorded after 24 h recovery. Corrected per cent mortalities (using Abbott's formula) after 1 h exposure and 24 h holding were used for assessing resistance status. To determine knock-down susceptibility,

*For correspondence. (e-mail: sks2000@vsnl.com)

RESEARCH COMMUNICATIONS

mosquitoes were exposed to 0.05% deltamethrin paper as mentioned above and the number of knocked down mosquitoes was recorded up to 120 min at every 5 min interval during the first hour and at 10 min interval during the second hour. For all susceptibility studies (mortality and knock down), the exposure tube was held in a vertical position during exposure as recommended by WHO¹⁶. The lethal times (LT₅₀ and LT₉₀) and knock down times (KDT₅₀ and KDT₉₀) were determined using log-time and probit-mortality regression model of Finney¹⁷. The susceptibility to DDT was determined by exposing mosquitoes to 4% DDT paper for 1 h and 24 h recovery. The data on temperature and relative humidity during experiments in field were also recorded. Mosquitoes collected from the study area were transported to our laboratory at Delhi. The susceptibility of 3 to 4-day-old and sugar-fed adult females from F₁ progeny were also determined against 0.05% deltamethrin as mentioned above under laboratory conditions.

The laboratory-reared *An. culicifacies* species B and C were used as reference susceptible mosquitoes. The per cent mortalities and knock-down times (KDT₅₀ and KDT₉₀) were determined for these reference mosquitoes as described above, except that the exposure time for mortality data against 0.05% deltamethrin was limited to 10 min.

To study the differential knock-down susceptibility of different members of the *An. culicifacies* complex prevalent in the study area, field collected semi-gravid females were exposed to 0.05% deltamethrin paper in exposure tubes for one hour and knocked down susceptible and resistant mosquitoes were separated immedi-

ately. Ovaries from these mosquitoes were removed immediately after exposure and preserved in modified Carnoy's fixative (1 : 3 glacialacetic acid and methanol) for sibling species identification. Polytene chromosome plates from ovarian nurse cells were prepared following Green and Hunt¹⁸ and sibling species identification was done on the basis of species-specific paracentric inversions seen on polytene chromosomes². To test the differential knock-down susceptibility among the members of *An. culicifacies*, chi-square test was performed.

The corrected per cent mortalities in *An. culicifacies sensu lato* from different areas after one hour exposure to WHO's diagnostic concentration of deltamethrin, i.e. 0.05% impregnated papers followed by 24 h recovery time and their respective LT₅₀ and LT₉₀ values are shown in Table 1. The per cent mortalities in mosquitoes collected from study villages ranged between 60.3 and 78 in five study villages, whereas reference susceptible *An. culicifacies* species B and C colonies showed 100% mortality at this concentration. There was no significant difference in susceptibility of wild-caught mosquitoes from regularly/occasionally SP-sprayed and unsprayed villages, all of which are located within a radius of 50 km. The LT₅₀ and LT₉₀ values of mosquitoes in sprayed villages were 27.3 and 164.5 min and in unsprayed villages 38.0 and 218.2 min respectively against 0.05% deltamethrin, whereas LT₉₀ of susceptible strain was < 10 min. The 50% (KDT₅₀) and 90% (KDT₉₀) knock-down times of field-collected *An. culicifacies* to 0.05% deltamethrin varied between 74.4–81.5 min and 219.7–296.8 min respectively. *An. culicifacies* in this area was found to be resistant to DDT and per cent corrected

Table 1. Susceptibility of *Anopheles culicifacies* to DDT and deltamethrin

Village (PHC, Taluka)	History of insecticides used	Deltamethrin 0.05%			DDT 4%
		Mortality	LT ₅₀ and LT ₉₀ (min)	KDT ₅₀ and KDT ₉₀ (min)	Mortality*
<i>Field-collected mosquitoes</i>					
Gangapore (Amladam, Mandavi)	1995: MAL 1996: DEL 1997 and 1988: CYF 1999 and 2000: DEL	60.4%* (n = 106)	LT ₅₀ : 38.0 LT ₉₀ : 218.2	KDT ₅₀ : 81.5 KDT ₉₀ : 230.5	13.1% (n = 74)
Limbi (Bareda, Sonegarh)	1997: DDT 1999 and 2000: DEL	61.3%* (n = 109)	–	KDT ₅₀ : 80.6 KDT ₉₀ : 219.7	6.9% (n = 58)
Kakrapar (Dadheda, Mandavi)	1999: DEL (ITMN)	78.3%* (n = 106)	–	KDT ₅₀ : 81.0 KDT ₉₀ : 296.8	6.7% (n = 60)
Nidwada (Bareda, Sonegarh)	Unsprayed	69.2%* (n = 117)	LT ₅₀ : 27.3 LT ₉₀ : 164.5	KDT ₅₀ : 74.4 KDT ₉₀ : 216.6	–
<i>F₁ progeny of field-collected mosquitoes</i>					
Nidwada		72.8%* (n = 59)	–	KDT ₅₀ : 63 KDT ₉₀ : 162	8% (n = 45)
<i>Laboratory-colonized pyrethroid susceptible strains</i>					
Species B (DDT-resistant)		100% [‡] (n = 100)	LT ₉₉ : < 10	KDT ₅₀ : 8.8 KDT ₉₀ : 14.2	3.66% (n = 300)
Species C		100% [‡] (n = 100)	LT ₉₉ : < 10	KDT ₅₀ : 10.7 KDT ₉₀ : 15.7	

*After 1 h exposure and 24 h holding; [‡]10 min exposure; MAL, malathion; CYF, cyfluthrin; DEL, deltamethrin; ITMN, insecticide-treated mosquito net.

mortality against 4% DDT ranged between 6.7 and 13.1. The temperature and relative humidity during experiments ranged between 27.6–29.0°C and 75–97% respectively. The corrected per cent mortalities of F₁ progenies of *An. culicifacies* collected from an unsprayed village (Nidwada) against deltamethrin (0.05%) and DDT (4%) were 72.8 and 8 respectively under laboratory conditions. The KDT₅₀ and KDT₉₀ values against 0.05% deltamethrin were 63 and 162 min respectively. Laboratory colonized pyrethroid-susceptible species B and C showed 100% mortality when exposed to 0.05% deltamethrin for even 10 min and the calculated KDT₅₀ and KDT₉₀ values were 8.8–10.7 and 14.2–15.65 min respectively.

Cytological examination of ovaries from knock-down resistant and susceptible mosquitoes, discriminated by one hour exposure to 0.05% deltamethrin paper, revealed that 51.2% ($n = 43$) of species B and 65.4% ($n = 26$) of species C were susceptible, indicating statistically no difference in susceptibility between the two species ($\chi^2 = 0.25$, $df = 1$, $P > 0.05$).

Synthetic pyrethroids have emerged as alternative insecticides for control of multiple resistant disease vectors globally, due to their rapid killing action at relatively low doses, less mammalian toxicity and the nature of degradability. These insecticides have been used for indoor residual spraying or for impregnation of mosquito nets successfully, and had been the only choice for mosquito net impregnation worldwide till recently. Besides the optimism that pyrethroids will not produce resistance because of their rapid killing action, resistance to pyrethroids is emerging worldwide in insects of medical and agricultural importance. Among malaria vectors resistance has been reported in *An. gambiae* s.s. in West Africa¹⁹, *An. albimanus* in Central America²⁰, *An. sacharovi* in Turkey²¹, *An. sinensis* in China²² and *An. funestus* in South Africa²³.

In India SPs were introduced in public health programmes in the 1990s to combat malaria epidemic and to control triple-resistant mosquitoes in certain localities. In Surat district, SP was introduced in 1996 and within five years of use of deltamethrin and cyfluthrin, *An. culicifacies* has developed resistance to pyrethroid. The level of resistance in *An. culicifacies* in this area may be considered high, because the present diagnostic dose (0.05% for 1 h)¹⁶ is considerably high for *An. culicifacies*, as evident by the fact that the laboratory-colonized strains are 100% susceptible to this dose when exposed for 10 min only. Because of high diagnostic dose, it is possible to miss early cases of resistance.

The available data on susceptibility of *An. culicifacies* in other parts of India where SP has been used or is being used do not show rapid development of resistance against SP. In Rameshwaram island (southern India), where species B and E are prevalent³ and deltamethrin is being used in the form of indoor residual spray, there was 100% mortality as measured against 0.025% deltamethrin

in 1997, after continuous spray since 1991. However, there was slight reduction in susceptibility to knock-down, KDT₅₀ and KDT₉₀ being 18.63 and 31.58 min against 0.05% deltamethrin. Later laboratory selection of this population for 12 generations resulted in 15-fold (LT₉₀) resistance to deltamethrin²⁴. In Ghaziabad (northern India) where *An. culicifacies* species A is prevalent, continuous spray of deltamethrin for three years did not result in resistance in this vector, although there was a sign of development of resistance in *Culex quinquefasciatus*²⁵. In Mewat, another area of northern India, where species A and B are sympatric with predominance of the former, deltamethrin spray during 1996–97 has not led to development of resistance in *An. culicifacies* (Raghavendra, K., unpublished data). In the present study area, species B and C are prevalent. Thus, these evidences suggest that the selection pressure has resulted in development of pyrethroid resistance in species B, C and E, and there is no indication of development of pyrethroid resistance in species A or D so far.

There are reports where resistance against DDT in *An. gambiae* confers cross-resistance to pyrethroid (*kdr*)²⁶. In *An. culicifacies* there is no evidence of cross resistance of DDT with pyrethroid. In a survey carried out in 1987 in Surat, this vector was reported to be resistant to DDT but was fully susceptible to deltamethrin, as determined against 0.025% deltamethrin²⁷. Similarly, a laboratory maintained DDT-resistant strain, which has never been exposed to any pyrethroid is highly susceptible to deltamethrin (Table 1). A definite conclusion on *kdr*-type resistance can be drawn by generating data on nerve insensitivity to pyrethroids or mutation in sodium channel gene in pyrethroid-resistant *An. culicifacies*.

The development of pyrethroid resistance in *An. culicifacies* is of great concern to the malaria control programme because SPs are being used in public health programmes to control multiple-resistant vectors and tackle epidemic outbreaks. Also, this is the only group of insecticides currently used for bed-net impregnation for malaria control. SPs are currently being used in many states in India not only for vector control, but also in the agricultural sector, mainly for control of cotton pests. The pyrethroids were introduced in Andhra Pradesh, as early as in 1980 for control of cotton pests and as a result *Helioptera armigera* developed 164 to 300-fold resistance to SPs by 1987–88 (ref. 28). Now this pest has developed a 5–6500-fold resistance against pyrethroids in all parts of India, particularly Maharashtra, Punjab, Andhra Pradesh and Tamil Nadu²⁹. Such a high level of resistance in this cotton pest indicates indiscriminate use of pyrethroids in the agricultural sector. Though there is no evidence of use of SPs in agricultural sector in the present study area, this should be investigated in other areas where SPs are being used for crop protection. In addition, the molecular and biochemical basis of pyrethroid resistance should be explored for its early detec-

tion and to understand the cross-resistance to other commonly used insecticides.

1. Sharma, V. P., *Curr. Sci.*, 1998, **75**, 1127–1140.
2. Subbarao, Sarala K., Vasantha, K. and Sharma, V. P., in *Bio-systematics of Haematophagous Insects*, Oxford University Press, 1988, pp. 25–37.
3. Kar, I. *et al.*, *J. Med. Entomol.*, 1999, **36**, 595–600.
4. Subbarao, S. K., Vasantha, K., Raghavendra, K., Sharma, V. P. and Sharma, G. K., *J. Am. Mosq. Control Assoc.*, 1988, **4**, 29–33.
5. Subbarao, S. K. *et al.*, *Trans. R. Soc. Trop. Med. Hyg.*, 1992, **86**, 613–614.
6. Adak, T., Kaur, S. and Singh, O. P., *ibid*, 1999, **93**, 573–577.
7. Kaur, S., Singh, O. P. and Adak, T., *J. Parasitol.*, 2000, **86**, 1345–1348.
8. Subbarao, S. K., Vasantha, K. and Sharma, V. P., *Med. Vet. Entomol.*, 1988, **2**, 219–223.
9. Raghavendra, K., Vasantha, K., Subbarao, S. K., Pillai, M. K. and Sharma, V. P., *J. Am. Mosq. Control Assoc.*, 1991, **7**, 255–259.
10. Raghavendra, K., Subbarao, S. K., Vasantha, K., Pillai, M. K. and Sharma, V. P., *J. Med. Entomol.*, 1992, **29**, 183–187.
11. Rahman, J., Roy, M. L. and Singh, N. N., *Indian J. Malariol.*, 1959, **12**, 125–130.
12. Pal, R., World Health Organization, Geneva, Mimeographed Report WHO/MAL/482.65, 1965.
13. Patel, T. B., Ramachandra Rao, T., Halgeri, A. V. and Deobhanekar, R. B., *Indian J. Malariol.*, 1958, **12**, 367–370.
14. Sharma, M. I. D. and Samnotra, K. G., *Bull. Nat. Soc. India Mal. Mosq. Dis.*, 1962, **10**, 151–157.
15. Rajagopal, R., *Indian J. Med. Res.*, 1977, **66**, 27–28.
16. World Health Organization, Geneva, WHO/CDS/CPC/MAL 98.12, 1998, (unpublished document).
17. Finney, D. J., *Probit Analysis*, Cambridge University Press, Cambridge, 1971, 3rd edn.
18. Green, C. A. and Hunt, R. H., *Genetica*, 1980, **51**, 187–195.
19. Vulule, J. M., Beach, R. F., Atieli, F. K., Mount, D. L., Roberts, J. M. and Mwangi, R. W., *Med. Vet. Entomol.*, 1994, **8**, 71–75.
20. Brodgon, W. G. and Barber, A. M., *Pestic. Biochem. Physiol.*, 1990, **37**, 130–139.
21. Kasap, H., Kasap, M., Alptekin, D., Luleyap, U. and Herath, P. R. J., *Bull. WHO*, 2000, **78**, 687–692.
22. Wang, J., *J. Am. Mosq. Control Assoc.*, 1999, **15**, 308–311; 2000, **16**, 9–12.
23. Hargreaves, K., Koekemoer, L. L., Brooke, B. D., Hunt, R. H., Mthembu, J. and Coetzee, M., *Med. Vet. Entomol.*, 2000, **14**, 181–189.
24. Mittal, P. K., Adak, T., Singh, O. P., Raghavendra, K. and Subbarao, S. K., *Curr. Sci.*, 2002, **82**, 185–188.
25. Ansari, M. A., Sharma, V. P., Razdan, R. K. and Mittal, P. K., *Indian J. Malariol.*, 1990, **27**, 1–13.
26. Chandre, F., Darriet, F., Manguin, S., Brengues, C., Carnevale, P. and Guillet, P., *J. Am. Mosq. Control Assoc.*, 1999, **15**, 53–59.
27. Raghavendra, K., Ph D thesis, Delhi University, Delhi, p 177.
28. Mueller-Beilschmidt Doria, *J. Pestic. Reform*, 1990, **10**, 34–38.
29. Armes, N. J., Jadav, D. R. and De Souza, K. R., *Bull. Entomol. Res.*, 1996, **86**, 499–574.

ACKNOWLEDGEMENTS. We thank Dr R. S. Yadav, MRC Field Station, Nadiad and Mr B. D. Patel, Surat for help during field visit and providing data on insecticide application in study villages. Thanks are due to Mr Krishan Gopal, Mr B. M. Sharma and Mr Uday Prakash for technical assistance.

Received 15 November 2001; revised accepted 26 December 2001

Once-a-month injection of norethisterone enanthate and estradiol valerate combination suppresses pituitary FSH/LH secretion and testicular function in man

K. M. Prasanna Kumar[†], H. Krishnamurthy[#],
H. N. Krishnamurthy[#], G. Shetty[#] and
N. R. Moudgal^{#,‡,*}

[†]Department of Endocrinology, M.S. Ramaiah Medical Teaching Hospital, MSRIT Post, Bangalore 560 054, India

[#]Department of Molecular Reproduction, Development and Genetics, Indian Institute of Science, Bangalore 560 012, India

[‡]Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur, Bangalore 560 064, India

The efficacy of 200 mg of norethisterone enanthate (NET-EN) and 2 mg of estradiol valerate (E-VAL) combination to suppress FSH/LH secretion and testicular function in adult volunteer men has been examined. The volunteer men received a single intra muscular injection of the hormone combination on days 1, 25, 50 and 75 of the study. On days 100, 125, 150 and 175 they received an injection of NET-EN alone. Within three days of first injection serum testosterone (T) became undetectable (indicates suppression in LH secretion) and FSH level was reduced by 85%. Maximal suppression in FSH and T levels lasted for 15 days, the levels returning to normal by 25–30 days of a single injection. The subjects were given every alternate day starting from day 30 of treatment an oral T supplement (40 mg T undeconate tablet). Seminal ejaculates obtained once in 15–20 days were analysed for sperm counts and motility. Sperm counts (expressed as million per ml or ejaculatory volume) showed significant drop in all treated men by day 54 (> 85%, $P < 0.05$) and reached acute oligo/azoospermia by day 110 and beyond. Motile sperm count showed drastic reduction from day 50 onwards (from 60.5 ± 13.7 mill/ml or 93.7 ± 23.7 mill/ejaculate before treatment to 1.5 ± 0.65 mill/ml or 2.4 ± 1.3 mill/ejaculate). Fertility index (FI), a product of motile sperm count and motility score, providing a measure of the fertilizing potential of sperm, was reduced from a pre-treatment control of 268 ± 55.1 to 4.85 ± 2.7 by day 54 of treatment. Administration of NET-EN alone from the 5th injection onwards was adequate to maintain blockade in sperm production till the end of the study. No signs of hepatotoxicity or change in lipid profile were observable as a consequence of treatment. The potential uses of such a convenient regimen in suppressing testicular function appear many and need to be exploited.

A variety of steroidal compounds have been tested for their efficacy to block pituitary gonadotrophin secretion

*For correspondence. (e-mail: moudgal@hotmail.com)