INSECTICIDE RESISTANCE STATUS AND MECHANISMS IN MALAYSIAN *BLATTELLA GERMANICA* (LINNAEUS)

HL Lee, WD Tien and Baharudin Omar

Division of Medical Entomology, Institute for Medical Research, Jalan Pahang, 50588 Kuala Lumpur; Department of Biomedical Sciences, Faculty of Allied Health Sciences, Universiti Kebangsaan Malaysia, Kuala Lumpur

Abstract. The insecticide resistance status of 4 strains of adult male *Blattella germanica*, viz M (Malacca), E (England), F (restaurant) and K (cafeteria) against malathion and bendiocarb compared with a reference susceptible strain (S) was determined by using a modified WHO bioassay method. The results indicated that all the 4 strains were resistant to the insecticides albeit in different degrees. Resistance ratios for malathion ranged from 1.85-41.07-fold, whereas that of bendiocarb ranged from 1.68-4.83-fold. The biochemical microplate enzyme assays technique employed indicated that the resistance in M and E strains were attributed to acetylcholinesterase insensitivity. Multiple resistance was not detected in any of the 4 strains. Parameters of the identified resistance mechanism correlated well with the observed level of resistance. Agar gel electrophoresis showed that variations in esterase isoenzymes did not confer organophosphate and carbamate resistance to the 4 strains.

INTRODUCTION

The cockroach today remains as one of the most important household pests in Malaysia (Yap *et al*, 1991). Its presence not only indicated unsatisfactory sanitary conditions, but it is also a mechanical vector of human diseases. In Malaysia, Anuar and Paran (1976) reported that *Periplaneta americana* is the intermediate host of *Moniliformis moniliformis* in the rat, while Rampal *et al* (1983) isolated *Salmonella typhi* and *Escherichia coli* from the intestines of several species of cockroaches collected from the household and hospital kitchens. The control of these dreaded insects is still dependent largely on the use of insecticides (Cornwell, 1968; Schal and Hamilton, 1990). The insecticides most commonly employed were those of the organochlorines, organophosphates, carbamates and recently synthetic pyrethroids. However, since 1953, cockroaches have been known to develop resistance to virtually all known insecticides (WHO, 1992). Many different mechanisms of insecticide resistance were also reported (Hemingway *et al*, 1993). In view of the lack of resistance information in Malaysian *B. germanica*, this study was conducted to investigate the status of resistance and to determine the mechanisms of resistance.

MATERIALS AND METHODS

Cockroach

Five strains of *B. germanica* were used in this study. Strain S is a susceptible strain reared in the insectarium of the Division of Medical Entomology, IMR for more than 10 years and is unexposed to any insecticides. Strain K and strain F were collected from a cafeteria and restaurant in Kuala Lumpur, respectively, while strain M was collected in Malacca and strain E originated from England and supplied by CCM Co, Malacca. All the strains were maintained in glass jars in the insectarium at 28°C at RH of more than 60%. Ground mouse chow was provided inside a small container and a moist cotton pad was also used to supply water.

WHO bioassay method

The WHO bioassay method for cockroach (1975) was used with slight modifications. Two insecticides, malathion and bendiocarb were first diluted in acetone and 2.5 ml of the solution was then pipetted into a glass jam bottle. The bottle was rotated slowly until the inner surface was coated evenly and all the acetone had evaporated. Bottles coated with acetone only were used as controls. The treated bottles were left overnight prior to testings. Two application dosages of malathion were tested, *ie* 10 mg/ft^2 for strain K and F and 20 mg/ft^2 for stain M and E. For bendiocarb, only 1 dosage, *ie* 2.5 mg/ft^2 was used against all strains. For a test, 10 adult male cockroaches were introduced into a treated bottle. The cumulative mortality of the cockroach was recorded at periodic inter-

vals until about 90% mortality was observed. Four replicates and a control was conducted in each test. The results were analysed using the probit analysis computer program of Raymond (1985) to obtain the lethal time values.

Non-specific esterases microassay

The microassay method was modified from *Lee et al* (1992). Briefly, the thorax of individual cockroach was severed using a scalpel and homogenized in 500 µl cold phosphate buffer (pH 7). The homogenate was pipetted into a microcentrifuge tube and spun at 8,500*g* at 4°C for 10 minutes. Fifty µl of the homogenates were transferred into each corresponding wells in the labeled microplate. Substrate solution was prepared by adding 0.5 ml alpha-naphthyl acetate in acetone (6g/ml) with 50 ml phosphate buffer (0.02M, pH 7.0). Coupling reagent was prepared by dissolving 8.75 *g* of sodium dodecyl sulfate and 0.75 *g* Fast Blue B salt in 50 ml of distilled water. Addition of 50 µl of substrate solution and then coupling reagent followed. Optical density readings at 410 nanometer wavelength from an Immunoassay Reader (Dynatech MR 5000) was taken 10 minutes after the appearance of the color reaction. Fifty µl of acetic acid solution was added to each of the wells to stop the reaction and to preserve the plates for color documentation. Optical density readings were pooled together for analysis.

Microassay of insensitive acetylcholinesterase

Homogenate from the cockroach was screened for insensitive Ache using a modification of Ellman test (Brogdon *et al*, 1988). The thorax of each individual was used for all experiments. The thorax was homogenized in wells of a plastic spot plate with 100 µl of 0.05 M solution of potassium phosphate buffer using a glass rod. Each homogenate was diluted to a final volume of 500 µl with the same buffer, transferred into microcentrifuge tubes and centrifuged at 4,800*g* for 10 minutes. Fifty µl of the clear homogenate was then pipetted into a well of microplate. In this way, a total of 8 replicates (50 µl) could be obtained from each cockroach for testing. A 50 µl aliquot mixture of 10% acetone-buffer solution of acetylthiocholine (ACTH) plus various concentrations of bendiocarb (inhibitor) was added into each test well. This was followed by the addition of a 50 µl aliquot of 5,5-dithiobis (2-nitrobenzoic acid) (DTNB). After incubation at room temperature (28°C) for 30 minutes the intensity of the chromophore was scored visually and scanned by an immunoassay reader (Dynatech, MR 600) at 410 nm. As a positive control, 50 µl ACTH solution (without bendiocarb) was used.

Determination of Ache level

The same procedures mentioned above for insensitive Ache was followed, except that no inhibitor was added.

Total protein determination

The total protein content of the homogenate was directly determined by the colorimetric method of Bradford (1976) using a commercial test kit (STRATAGENE).

Agar gel electrophoresis of esterases

To visualize isoenzymes (carboxyesterases) responsible for the resistance of the cockroach, agar gel electrophoresis (Tadano, 1986) was conducted. Gel was prepared by dissolving 0.75*g* agarose and 2*g* polyvinyl pyrolidone in 100 ml of phosphate buffer solution (75 ml 0.063M $KHPO_4$ and 25 ml distilled water). Continuous swirling while heating was made to obtain even suspension. After agarose was completely dissolved, it was poured onto a glass mold (17 cm × 18 cm) placed on top of a hot plate to prevent immediate setting which would result in uneven thickness and spread of the gel on the mold. Individual males were homogenized in 5 small drops of 0.1% Tween 20. A small piece of filter paper (1 mm × 2 mm) was used to absorb the crude homogenate. Filter papers with homogenates were then lined up on the gel origin for absorption of the homogenate into the gel. Samples were horizontally electrophoresed at 4°C at 20 m A°/18 cm for 2 hours. Alpha and beta naphthyl acetate substrate solutions were used to detect esterases present in the mosquito. Substrate (0.75 *g* alpha-naphthyl acetate and 0.75 *g* beta-naphthyl acetate dissolved in 60 ml acetone with 25 ml distilled water) solution was sprayed and incubated at 37°C for 30 minutes. Five ml 1% stain solution (Fast Blue B salt in distilled water) was then poured onto

the gel and incubated for 15 minutes at 37°C. Stained gels were washed with tap water to remove excess stain. Gels were placed back into the incubator for drying for about 24 hours. Gels were compiled and photographed for documentation. Electrophoretic mobility of the isoenzymes were measured. Frequency of the appearance of the fast allele alone, slow allele alone and both alleles together in one insect was counted. Intensity of the stained bands were compared visually.

RESULTS

Insecticide susceptibility

The result are shown in Tables 1-2. In Table 1, strain Sa was the reference strain exposed to malathion at 10 mg/ft² while Sb was exposed to 20 mg/ft². As such, the LT_{50} values of the cockroach exposed to different application rates were compared with strains exposed to the corresponding application rates. Strain M appeared to be the most resistant in comparison with strain Sb (resistance ratio at LT_{50}; RR_{50} = 41.07) exposed to 20 mg/ft²; and strain F was most resistant compared with Sa (RR_{50} = 2.36). Exposure to bendiocarb indicated that strain M was the most resistant to this insecticide at RR_{50} = 4.83.

Non-specific esterases determination

The level of non-specific esterases (NSE) was shown in Table 3. Two strains (F and K) exhibited elevated level of NSE compared with the reference strain; while NSE level in strains M and E was lower. The NSE levels in all these strains were significantly different from the reference strain ($p < 0.01$).

Acetylcholinesterase level

There was no significant difference in the level of acetylcholinesterase (Ache) found in the different strains of the cockroach ($p > 0.05$) (Table 3).

Insensitive Ache: In all strains except M and E, the activity of Ache was suppressed correspondingly when the concentration of the inhibitor (bendiocarb) increased (Fig 1). The Ache activity of strain M and E was suppressed initially, but the activity subsequently stabilized although the inhibitor concentration was increased to 1.25, 1.75 and 2.5 g/l.

Table 1

Susceptibility of *Blattella germanica* to malathion.

Strain	LT_{50}(min) (95% CL)	LT_{90}(min) (95% CL)	*RR_{50}	Regression
Sa	32.96 (32.21-33.71)	43.18 (41.59-45.28)	1	Y = 10.92X-120.78
Sb	17.35 (16.94-17.79)	23.49 (22.48-24.85)	1	Y = 9.74X-104.49
K	60.99 (57.97-64.22)	125.22 (113.68-141.33)	1.85	Y = 4.10X-43.35
F	77.69 (70.45-85.56)	220.69 (188.78-269.29)	2.36	Y = 2.83X-28.34
E	679.04 (662.09-696.30)	956.59 (913.99-1,012.95)	39.14	Y = 8.61X-105.51
M	712.54 (683.64-742.86)	1,291.88 (1,198.90-1,415.83)	41.07	Y = 4.96X-58.75

*RR_{50} = LT_{50} (Reference)/LT_{50}(Test)

Table 2

Susceptibility of *Blattella germanica* to bendiocarb.

Strain	LT_{50}(min) (95% CL)	LT_{90}(min) (95% CL)	*RR_{50}	Regression
S	20.64 (19.93-21.35)	31.38 (29.71-33.63)	1	Y = 7.04X-74.70
K	33.82 (32.19-35.43)	66.59 (61.75-73.00)	1.64	Y = 4.36X-45.22
F	46.27 (43.99-48.66)	96.89 (88.62-108.03)	2.24	Y = 3.99X-41.59
E	85.43 (80.61-90.44)	186.67 (169.52-210.06)	4.14	Y = 3.78X-40.06
M	99.60	202.49	4.83	Y = 4.16X-44.92

*RR_{50} = LT_{50} (Reference)/LT_{50}(Test)

Table 3

Non-specific esterases and acetylcholinesterase activity of *Blattella germanica.*

Strain	Mean esterases activity ± SD (alpha-naphthol/min/mg protein)	Mean Ache activity ± SD (activity/min/mg protein)
F	4.49 ± 0.34	0.97 ± 0.12
K	4.30 ± 0.20	1.03 ± 0.11
S	3.89 ± 0.25	1.06 ± 0.15
M	3.21 ± 0.46	1.09 ± 0.12
E	2.94 ± 0.30	1.11 ± 0.15

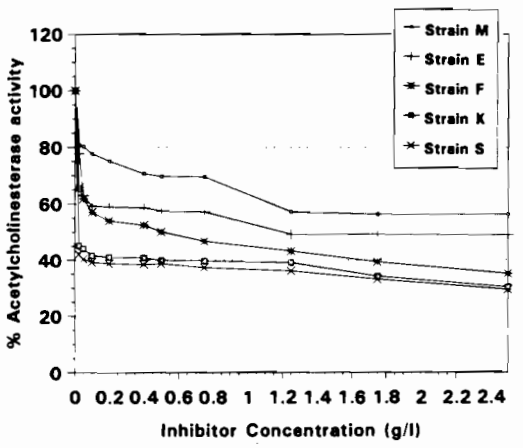

Fig 1–*In vitro* inhibition of acetylcholinesterase of different strains of *Blattella germanica* by bendiocarb.

Electrophoresis of esterases:

Two to three esterase loci in the various strains were determined from electrophoresis (Table 4). The most resistant strain exhibited 3 loci *ie* Locus I, II and III in which Locus I was heterozygous; while in the reference strain, similar pattern was observed. Hence it is clear that the variations occurred only between the individuals and not between the strains.

DISCUSSION

Field strains of German cockroach collected from Malacca, Malaysia were highly resistant to malathion (RR_{50} > 40) and showed a low degree of resistance to bendiocarb (RR_{50} < 5). The develop-

Table 4

Esterase isoenzymes patterns observed in individuals of different strain of *Blattella germanica*.

Types of locus	Strain				
	M	E	F	K	S
Locus I(F) + Locus II + Locus III	√	√	√	√	√
Locus I(S) + Locus II + Locus III	-	√	-	-	-
Locus I(F + S) + Locus II + Locus III	√	√	-	√	√
Locus I(F) + Locus II	√	√	-	√	√
Locus I(S) + Locus II	-	-	-	-	-
Locus I(F + S) + Locus II	-	-	√	-	-

ment of high resistance of this strain to malathion is unlikely due to the use of malathion, as this insecticide is not widely used for cockroach control in Malaysia. Malathion is mainly used in the dengue vector control program since 1973 in Malaysia. The fogging activity using this insecticide may expose other insects to its selection pressure resulting in the emergence of resistance. In this respect, Lee (1990) and Lee *et al* (1992) reported high resistance to malathion in larvae and adults of *Culex quinquefasciatus*. On the other hand, Nazni *et al* (1996) found very high resistance to malathion (RR_{50} > 10,000) in housefly from the Cameron Highlands; an agricultural area with intensive use of agrochemicals which include malathion for the control of pests. Thus in the process of insecticide application, non-target insects may also be selected for resistance due to the spill-over effects of spraying. Low level of resistance to bendiocarb may be due to the absence of this compound in household insecticides.

Resistance to organophosphate in strain M and E was not due to NSE since these strains exhibited low NSE activity, while highly susceptible strains of F and K showed high NSE activity. In addition, esterase electrophoresis patterns indicated that variations in isoenzyme was not correlated with resistance. The resistance is also not due to level of Ache since all strains exhibited similar level of activity. There are evidence, however, to suggest that insensitivity of Ache to inhibitors may account for the high resistance to OP in German cockroach. In strains M and E, the Ache activity was not inhibited further and stabilized when the inhibitor concentration was increased to 1.25-2.5 g/l, while for other strains, the Ache activity was further inhibited with increasing inhibitor concentrations. It is therefore obvious that the mechanism of OP resistance in strain M and E was attributed to Ache insensitivity. Hemingway *et al* (1993) similarly reported that the mechanisms of resistance in several geographical strains of German cockroach to OP and carbamates were those of insensitive Ache and elevated level of NSE. According to Hemingway *et al* (1993), insect resistance due to altered Ache may be categorised as that of high OP and low carbamate resistance or high carbamate and low OP resistance. Hence, the altered Ache-based resistance in Malaysian German cockroach falls into the first category. They also noted that mixed-function oxidases also involved in the detoxification of xenobiotics and that glutathion S-transferase did not play any role in resistance. The contributory role of these two enzymes to insecticide resistance in Malaysian *B. germanica* remain to be studied.

ACKNOWLEDGEMENTS

The authors thank the Director, Institute for Medical Research for permission to publish. Work mentioned in this paper received partial financial support from a Malaysian Government R and D Grant.

REFERENCES

Anuar AK, Paran TP. *Periplaneta americana* (L) as intermediate host of *Moniliformis moniliformis* (Bremser) in Penang, Malaysia. *Southeast Asian J Trop Med Public Health* 1976; 7 : 415-6.

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; 72 : 248-54.

Brogdon WG, Beach RF, Stewart JM, Castonaza L. Microplate assay analysis of the distribution of organophosphate and carbamate resistance in Guatemalan *Anopheles albimanus*. *Bull WHO* 1988; 66 : 339-46.

Cornwell PB. The cockroach: a laboratory insect and an industrial pest. Vol 1. London : Hutchinson 1968.

Hemingway J, Small GJ, Monro AG. Possible mechanism of organophosphorus and carbamate insecticide resistance in German cockroaches (Dictyoptera : Blattelidae) from different geographical areas. *J Ecol Entomol* 1993; 86 : 1623-30.

Lee HL. A rapid and simple biochemical method for the detection of insecticide resistance due to elevated esterase activity in *Culex quinquefasciatus*. *Trop Biomed* 1990; 7 : 21-8.

Lee HL, Abimbola O, Inder Singh K. Determination of insecticide susceptibility in *Culex quinquefasciatus* Say adult by rapid enzyme microassays. *Southeast Asian J Trop Med Public Health* 1992; 23 : 458-463.

Nazni WA, Lee HL, Yahya A, Sadiyah I, Tadano T. Current resistance status of the house fly, *Musca domestica* (L.) in Cameron Highlands, Malaysia. *Int Med Res J* 1996 (in press).

Rampal L, Pakeer Oothuman, Jeffrey J, *et al.* Bacterial pathogens from the intestinal tracts of various species of cockroaches. *Med J Malaysia* 1983; 38 : 104-7.

Raymond M. Log-probit analysis basic programme of microcomputer. *Cah ORSTOM Ser Entomol Med Parasitol* 1985; 23 : 117-21.

Schal C, Hamilton RL. Integrated suppression of synanthropic cockroaches. *Ann Rev Entomol* 1990; 35 : 521-51.

Todano T. Method for agar gel electrophoresis of various enzymes in mosquitoes. *Akaieka News* 1986; 10 : 100-6.

World Health Organization. Tentative instruction for determining the susceptibility or resistance of cockroaches to insecticides. Monograph of WHO/VBC/ 75. 593, 1975.

World Health Organization. Vector resistance to pesticides. Fifteenth report of the WHO Expert Committee on Vector Biology and Control. *WHO Tech Rep Ser* 1992; 818.

Yap HH, Chong NL, Loh PY, *et al.* Survey of domiciliary cockroaches in Penang, Malaysia. *J Biosci* 1991; 2 : 71-5.