## Original Article

# Level of Pyrethroid-resistance Associated with Cytochrome P450 Expression in German Cockroach *Blattella germanica* (Blattodea: Ectobiidae) in the Field Collected Strains

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## Abstract

**Background:** Cytochrome P450-dependent monooxygenases are a very important metabolic system involve in insecticide resistance. This study was conducted to find the association between the expression level of cytochrome P450 (CYP450) and permethrin-resistance level among four strains of the German cockroach *Blattella germanica* (L) (Blattodea: Ectobiidae).

**Methods:** Three field strains of German cockroach with different frequency of exposure to pesticides, and a laboratory susceptible strain were used in the present study. Insecticide susceptibility bioassays were carried out to detect resistance to permethrin. The concentration of CYP450 in each strain was determined using ion-exchange HPLC chromatography. Biochemical assays was performed to analyse CYP450 activities.

**Results:** The resistance ratios (RR) to permethrin among three field strains were 3.29, 4.10 and 6.17-fold comping with the susceptible stain. The CYP450 activity of three field strains was 1.6, 2.4 and 2.7 times higher than in the susceptible stain. The amount of CYP450 per mg of protein was significantly different between the susceptible and the three resistant strains. The resistant cockroaches showed a relatively high expression of CYP450 enzymes. A strong correlation was found between permethrin resistance level and total concentration of CYP450 enzymes.

**Conclusion:** The results of current study show that more frequent usage of a pyrethroid insecticide cause the metabolic insecticide resistance to rise in German cockroach. Therefore, there is a ratio correlation between resistance level and monooxygenases activities in insect. Thus, the control program must be managed according to history of pesticide usage.

**Keywords:** Cytochrome P450; German cockroach; Insecticide resistance; Permethrin

## Introduction

The German cockroach, *Blattella germanica* (L.) (Blattodea: Ectobiidae), is one of the major pests of public health importance. It is a vector of various bacterial and viral diseases (1, 2) and can induce allergic responses such as asthma in humans (3-5). Over the past years, the German cockroach has developed resistance to a wide range of classes of insecticides through multiple resistance mechanisms (6-9) and this has been a major barrier in the control of this pest (7, 10). Cytochrome P450 monooxygenases (CYP450) have been shown to be one of the most essential enzymes in the machinery of insecticide resistance, and play a crucial role in the metabolism and structural changes of endogenous and exogenous compounds (11) such as pesticide detoxification (12). It has been shown that, monooxygenase enzymes play a significant role in the German cockroach's resistance to pyrethroids (13-15). The CYP450 complex has a hemoprotein structure and ubiquitous enzymes. It is found in living organisms such as bacteria, plants, animals, and fungi. The CYP450 enzymes are involved in both endogenous and xenobiotic metabolic processes that occur within living organisms. In insects, these



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enzymes are necessary for the synthesis and degradation of steroid molting hormones, juvenile hormones and pheromones and also act as terminal oxidase in the monooxygenase system (16). The level of expression of cytochrome P450 can vary at different stages of insect development. Also, it has been found that there is a considerable diversity in the number of cytochrome P450 genes among insect species (17). The variation in gene expression may be due to the differences in response to inducers and the tissues in which they are expressed (18, 19). A study on the levels of resistance to permethrin and deltamethrin among three strains of the German cockroach collected from Alabama revealed that P450 monooxygenases are highly involved in pyrethroid resistance. Although these strains were collected from the same geographic location, their resistance mechanisms to pyrethroid were different(20). In a previous study, the levels of German cockroach resistance to pyrethroid and cross-resistance to DDT in seven field-collected strains were investigated. The synergists PBO (piperonyl butoxide) and DEF (*S*, *S*, *S*-tributylphosphorotrithioate) were found to play a role in permethrin resistance whereas chlorphenetol decreased DDT resistance in the seven collected insect populations (9, 21). Another bioassay study was conducted in 2007 to investigate the resistance level of field strains of the German cockroach to pyrethroids in the north of Iran. In the study, the activity of Glutathione S-transferases (GSTs), esterase and the monooxygenases of cytochrome P450 was assayed using ELIZA test and the resistance ratios of the field-collected strains ranged between 1.5 to 2.5-fold, indicating a higher tolerance in the German cockroach to pyrethroids. The authors suggested that the high resistance of the field-collected German cockroach in the area might have been caused by the extensive use of pyrethroids for pest control (22). In-depth knowledge on the role of cytochrome P450 in pesticide resistance in insects may provide imperative information for the development of novel pesticides for effective pest control. However, several studies have been conducted on pesticide resistance involving P450 in German cockroaches. The aim of the present study was to measure the content of CYP450 enzymes in the German cockroach population and to determine its correlation with the level of pesticide resistance among the different strains of this pest. The results of current study are important for the further understanding of the role of CYP450 in pesticide resistance mechanism.

## Materials and Methods

### Cockroach strains

Three field-collected and one susceptible strains of the German cockroach were used in the present study. The field strains were collected from the following sites: a restaurant with insecticide application once or twice a year (strain A), Sina hospital which has insecticide spraying activity more than twice a year (strain B), and Shariati hospital (strain C), where pesticides are frequently and heavily used. Pyrethroids (permethrin and cypermethrin) were the most common insecticides used in the insect collection sites. The field collected populations were colonized in insectary and $F_1$ generations were used for all experiments. The susceptible strain has been reared in the laboratory without exposure to insecticides since 1975. All cockroach strains were maintained in an insectary condition at 27±2 ºC and 60±10% RH, with a photoperiod of 12:12h (L: D). All strains were provided with unlimited bread, sugar and water. Only one to two-week old males with relatively homogenous physiology and body mass were used in this study. Cockroaches were anesthetized briefly with $CO_2$ to facilitate handling in each test.

### Insecticide resistance bioassays

Bioassay were carried out with permethrin (92%, technical grade) cis:trans 60:40, (Cyan-



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amid Agro, India). Glass jar method was used according to the previous study (23). A range of serial concentrations of permethrin (technical grade) were prepared in acetone and were exposed to the insects to find a discriminating dose for the male cockroaches. In a series of knockdown experiments, a concentration of 15mg/$m^2$ permethrin at 35min was found to be a discriminating dose for distinguishing the susceptible strain (SS) from the resistant strain (RR). Subsequently, the discriminating dose (15mg/$m^2$) was used to coat the glass jars. Jars were rotated gently in a hood until the acetone completely evaporated. Exposure times for each strain were computed at the discriminating dose to find the minimum (T1: to kill 5% of cockroaches) and maximum (Tn: to kill 95% of cockroaches) time of exposure. The tests were performed in 4 replicates each with 10 male adult cockroaches. The cockroaches were kept for 24h and were monitored for mortality. The knockdown time ($KT_{50}$) was measured and subjected to probit analysis.

**Chromatography assays**

Total CYP450 was purified as explained by Scharf et al. (1997) (13). Thirty adult male cockroaches from each strain were dissected, and their body contents (except gut contents) were transferred into eppendorf tubes and homogenized in 0.5ml homogenization buffer (sodium phosphate 0.1M pH 7.5). The homogenates were centrifuged at 11000g for ten minutes. Subsequently, the supernatants were centrifuged at 106000g for 60min (L5-50 bechman ultracentrifuge). The microsomal pellets were resuspended in 20µL resuspension buffer (homogenization buffer containing glycerol 30% [v/v]). Then, 40µL of solubilizing buffer (10mM sodium phosphate, 0.6% (w/v) sodium cholate, 1mM EDTA, 20% (v/v) glycerol, pH 7/4) was added to the yield and mixed gently by vortexing or petting on ice for 30 minutes. The mixture was then centrifuged at 106000g for 60 minutes at a degree of 4 °C to remove particulate matter. The supernatants were analyzed using high performance liquid chromatography (Knauer, Germany). Anion-exchange analytical HPLC column (Shodex Asahipack) was equilibrated with buffer A (20mM Tris/ acetate containing 1mM EDTA, 20% (v/v) glycerol). In order to obtain a standard chromatogram, 20µl of recombinant expiration Cytochrome P450 Reductase human (Purchased from Sigma Co.) was loaded onto the column. Subsequently, 20µl of the solubilized extracted microsomes from each sample was loaded onto the column with a 20µl super loop. Flow rate was measured as 1.0ml/min and optical density column effluent was detected at 350nm. A linear gradient of buffer B (buffer A + sodium acetate 0.8M) relative to buffer A (0-90%) was specifically used to elute cytochrome P450 proteins. The amount of CYP450 of each samples was measured with Chrome Gate software.

**Biochemical assays**

The activity of total CYP450 was measured based on the methods described by Penilla et al. (2007)(24). Briefly, twenty microliters of the insect homogenate was aliquoted prior to the addition of 80µl of 0.625M potassium phosphate buffer (pH 7.2), 200µl of 3,3′,5,5′-Tetramethylbenzidine (TMBZ) solution (0.01g TMBZ dissolved in 5ml methanol plus 15ml of 0.25M sodium acetate buffer, pH 5.0) and 25µl of 3% hydrogen peroxide to each of a 96-well microtiter plate. The plates were incubated at room temperature for a period of 2 hours and their absorbances were measured at 450nm as an endpoint in the plate reader, and the values were compared with a standard curve of recombinant expiration Cytochrome P450 Reductase human (Purchased from Sigma Co.)

**Statistical analysis**

Cockroach knockdown data were assessed by probit analysis (Throne et al. 1995) using SPSS. Log-probit analysis was used to determine the lethal time required to knockdown



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50% of a population ($KT_{50}$) and the 95% confidence interval (CL). Resistance ratios were calculated as the $KT_{50}$ of resistant strains divided by the $KT_{50}$ response value of the SS strain. Linear regression of probit knockdowns was analyzed using SSPS. The strains were compared using the ANOVA; Games-Howel test ($p< 0.01$, and $p< 0.001$).

## Results

### Determination discrimination concentration

In a series of knockdown experiments, a concentration of 15mg/m$^2$ permethrin at 35min was found to be a discriminating dose for distinguishing the susceptible strain (SS) from the resistant strain (RR).

### Insecticide resistance bioassays

The results of insecticide resistance bioassays are represented in Table 1. The resistance ratio at $KT_{50}$ was highest in Strain C (6.17-fold) which had the highest exposure to pesticides followed by strain B and strain A with resistance ratios of 4.10 and 3.29-fold, respectively. The probit regression analysis of knockdown times of permethrin for the susceptible strain (SS) and field-collected strains (A, B and C) are shown in Fig. 1. The probit regression shows time-to-knockdown for the four strains. A high variation in time-to-knockdown was observed among the resistant strains (A, B and C), correlating with the degree of exposure to the pesticide. Strain C had the highest resistance to permethrin, with $KT_{50}$ and $KT_{95}$ values of 52.21 and 79.76 minutes, respectively. The $KT_{50}$ and $KT_{95}$ values of strain B were 34.72 and 55.88 minutes, respectively, whereas, the $KT_{50}$ and $KT_{95}$ values for stain A were 27.79 and 44.00 minutes respectively. The resistance ratios (RR) of the three field-collected cockroaches, strains A, B and C were 3.29, 4.10 and 6.17-folds, respectively, when compared with the susceptible strain (Table 1).

### Biochemical assays

The result of biochemical assay has been presented in Table 2. The data presented herein show a higher CYP450 activity in the strains C and B compared with the susceptible strain. The CYP450 activity in the resistant strains was 1.6 (strain A), 2.4 (strain B) and 2.7-fold (strain C) higher than in the susceptible stain (SS). These results indicate that the monooxidase activities between strains are statistically difference ($p< 0.001$).

### Chromatography assays

The analysis of high-performance liquid chromatography indicated that the amount of CYP450 significantly varied between four strains. The average of CYP450 concentration obtained from susceptible, A, B, and C strains were 0.417, 0.738, 1.110 and 1.302ng/mg respectively (Fig. 2). In comparative perspective of the CY450 expression and $KT_{50}$ ratio, the enzyme concentration enhanced with increasing resistance levels to insecticides among strains (Fig. 4).

**Table 1.** Results of probit analysis of time of knockdown response of four German cockroach strains to permethrin

| strain | Y-intercept | B±S.E. | $X^2$ (DF) | $KT_{50}$ | $KT_{90}$ | RR[a] |
|---|---|---|---|---|---|---|
| **SS** | -1.297 | 0.15±0.21 | 4.72(5) | 8.46 | 16.82 | 1.00 |
| **A** | -2.157 | 0.79±0.10 | 7.66(5) | 27.79 | 44.00 | 3.29 |
| **B** | -2.103 | 0.61±0.10 | 6.01(5) | 34.72 | 55.88 | 4.10 |
| **C** | -3.251 | 0.57±0.21 | 1.63(5) | 52.21 | 79.76 | 6.17 |

Susceptible strain (SS) is the susceptible laboratory strain; strains A-C are the field-collected strains.
Df= Degrees of freedom, KT= Knockdown time, $X^2$= chi-square.
[a] RR (resistance ratio) = $KT_{50}$ of field-collected strains / $KT_{50}$ of susceptible strain.
B =Unstandardized coefficients, S.E= Standard error.



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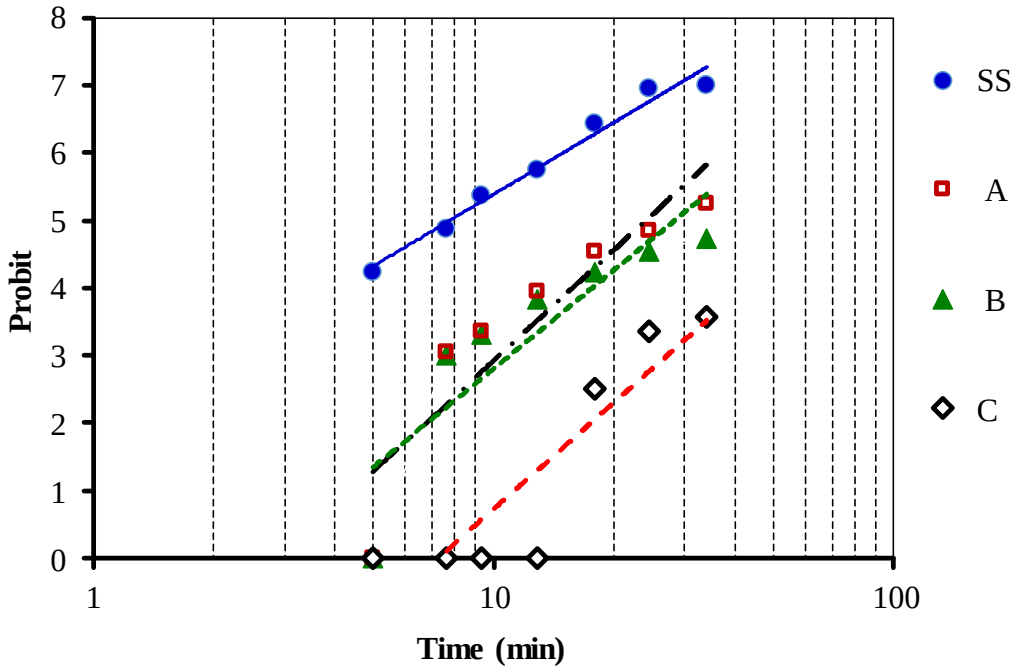


**Fig. 1.** Probit analysis of knockdown time of permethrin at the discriminating dose (15mg/m$^2$) for the four strains of the German cockroach. Strain SS is the susceptible laboratory strain; strains A, B and C are the field-collected strains

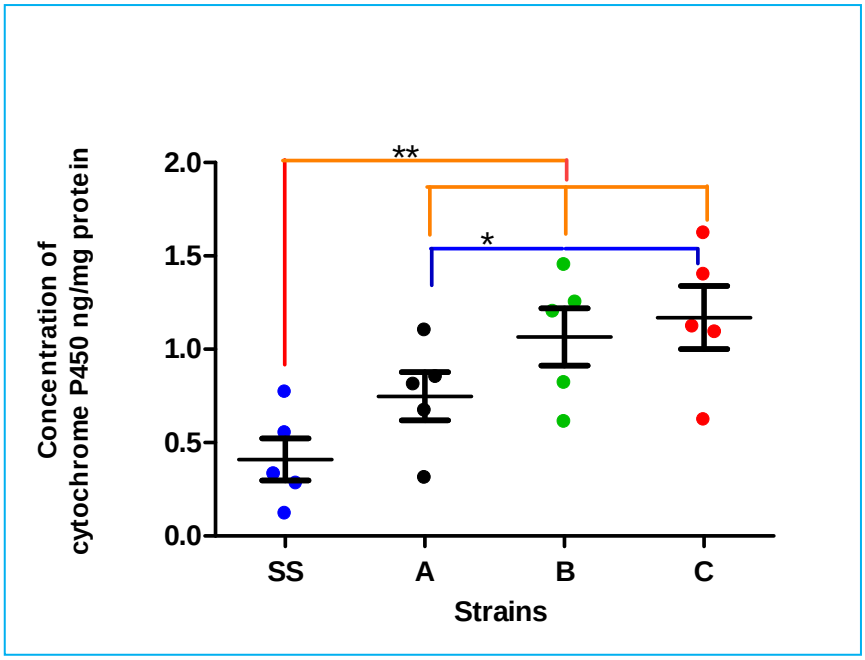


**Fig. 2.** Relative concentration of cytochrome P450s in susceptible strain (SS) and permethrin-resistant strains (A, B and C) of German cockroach *(Blattella germanica)*. The error bars represent means ± standard deviation. The enzyme concentrations were determined by HPLC. The strains were compared using the ANOVA; Games-Howel test, *p< 0.01, **p< 0.001 indicate significant difference



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**Table 2.** Descriptive analysis of the results of biochemical assays performed on German cockroach strains

| Strain | Mean (units/mg protein) | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Min | Max |
|---|---|---|---|---|---|---|---|
| | | | | Lower Bound | Upper Bound | | |
| **SS** | 0.48 | 0.03 | 0.01 | 0.44 | 0.53 | 0.46 | 0.52 |
| **A** | 0.76 | 0.04 | 0.02 | 0.69 | 0.83 | 0.72 | 0.81 |
| **B** | 1.17 | 0.02 | 0.01 | 1.14 | 1.19 | 1.15 | 1.19 |
| **C** | 1.30 | 0.07 | 0.04 | 1.18 | 1.42 | 1.24 | 1.37 |

Susceptible strain (SS) is the susceptible laboratory strain; strains A-C are the field-collected strains. No. of repetition for each strain = 4

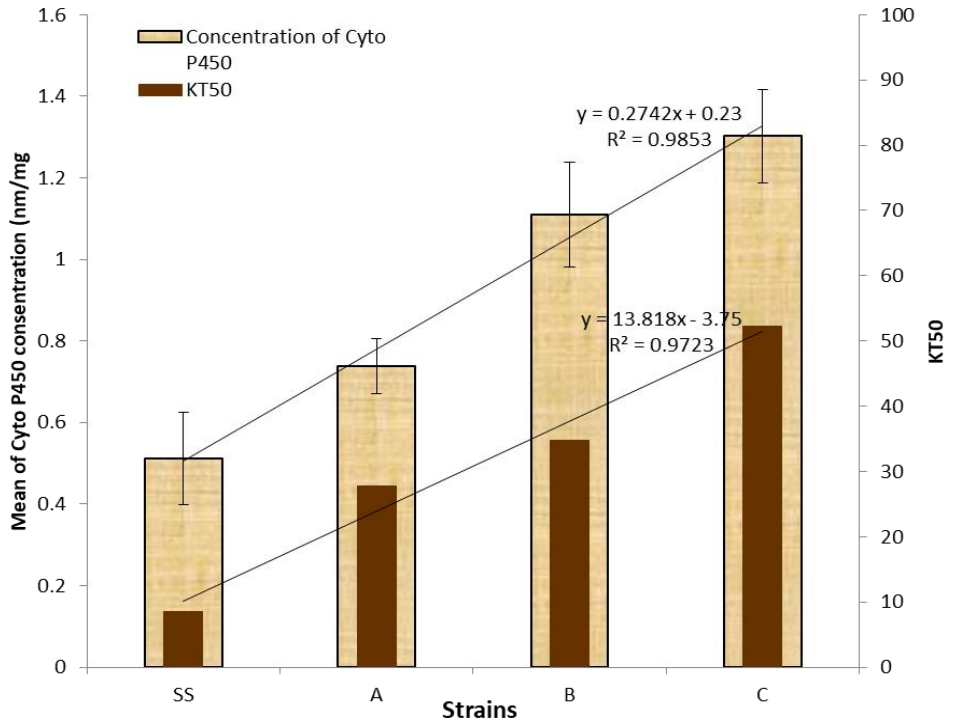


**Fig. 3.** Comparison of CYP450 expression and $KT_{50}$ between the susceptible strain (SS) and the three permethrin-resistant strains (A, B and C) of the German cockroach (*Blattella germanica*). $KT_{50}$: knockdown time of different strains of the cockroach exposed to permethrin 15mg/$m^2$.

## Discussion

Resistance to insecticides among insects has been associated with enhanced enzymatic metabolism, particularly increased activity of cytochrome P450s (25). Insect CYP450 complex play an important role in the detoxification of insecticides, which can lead to increase in insecticide resistance. There is little information on the association between the level of insecticide large peak in strains B and C. These results indicate that a higher modification occurred in the expression of CYP450 which correlated with increased level of permethrin resistance in the field-collected strains. The CYP 450 content also varied between the resistant and the susceptible cockroaches (Fig. 3). The highest CYP450 content was found in strain C followed by strain B. In addition, the CYP450 content in strain A was significantly different


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from that of strain S (Fig. 2). Fig. 3 shows the relationship between the level of permethrin resistance and CYP450 concentration in the German cockroach strains. Increased CYP450 content correlated resistance and the amount of enzyme expression in insects. In the present study, the amount of total of CYP450 in four strains of the German cockroach with different levels of susceptibility to permethrin was investigated in order to determine the association between resistance to insecticide and monooxygenase enzymes expression in this pest.

Higher level of expression of CYP450 protein is associated with excessive expression of P450 genes in insecticide-resistant insects (26). Similarly, it has recently been reported that there is alteration in the pattern of expression of some important proteins associated with detoxification in pyrethroid-resistant German cockroaches. These proteins include energy-metabolism proteins, detoxification-related proteins, signal molecule-regulated proteins, kinetic-related proteins, and gene expression-related proteins. The authors also stated that the in the pattern of gene expression is associated with stress induced by the pesticide (27). It has been shown that the regulation of multiple P450 genes through both constitutive overexpression and induction mechanisms are co-responsible for permethrin resistance in mosquitoes including *Culex quinquefasciatus*. Both mechanisms provide additional metabolic support for the detoxification of Permethrin, which in turn, may lead to the development of resistance (28). Moreover, a previous study indicated that 8 CYP genes are responsible for CYP450 expression in *Culex pipiens* complex (29). In the present study, we also found different concentration of CYP450 protein based on HPLC chromatograms analyzing (Fig. 2). The data suggest that those cockroach strains that were more resistant to permethrin, the level of expression of total CYP450 elevated (Fig. 3). These evidences suggest that several genes are involved in the expression of CYP450 enzymes. This also indicates that the German cockroach can develop a complicated mechanism of resistance to insecticides over time and place. In the present study, CYP450 content significantly increased with increasing level of insecticide resistance (Fig. 3). The resistance ratio in all the field-collected resistant strains was more than three-fold when compared with the susceptible strain (Table 1), which correlated with an increase in the concentration of enzymes and $KT_{50}$ (Fig. 3). However, the concentration of enzyme and KT increased with relatively similar slope (Fig. 4). This results may indicate a positive correlation exist between CYP 450 consecration and resistance level. The German cockroach strains were collected from three different locations in Tehran city, but the expression pattern of CYP450 and the level of resistance of the three strains to permethrin were directly associated with frequency of insecticide use in the collection site. German cockroaches have a high potential in developing resistance towards chemical insecticides but the pattern of insecticide resistance may vary based on the geographical location. In the present study, we collected the cockroach strains from three different locations in Tehran city with different frequency of permethrin application and we observed that the pattern of resistance to permethrin was quite different. Thus, the use of a particular insecticide against the German cockroach on a larger scale may not be effective, and the frequency of insecticide use should also be considered even for the same area.

Some studies have also suggested that resistance to insecticides depends not only on the evolutionary adaptation towards insecticide pressure but also the biological conditions of the location where the cockroaches inhabit is important (30). Hence, chemical control failure can probably occur in any setting as a result of insecticide resistance due to other biological factors. German cockroaches with great adaptive potential for resistance to insecticides may be scattered over a small area in urban /rural places. Therefore, a similar insecticide prescrip-



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tion may not be useful on a larger scale, and an integrated resistance management programs for combating the German cockroach must be considered even within small areas.

## Conclusion

CYP450 enzymes' expression are the successful adaptation of many insect pests to protect them against insecticides. It is clear that the enhanced metabolism of the insecticide in the resistant strains of the pests is associated with the expression of various CYP450 enzymes (31). In the present study, we observed more than one cytochrome P450 isozymes which were involved in the pyrethroid (permethrin) resistance. In addition, insecticide resistance level depends highly on the biological conditions of the insect collection sites, therefore, resistance monitoring should be done based on the specific location where the cockroaches inhabit. It can be concluded that German cockroach strains, even those from the same geographic source, can develop diverse mechanisms of resistance. Therefore, alternative chemical or nonchemical insecticides should be considered as a part of the integrated pest management (IPM) based on the insecticide resistance status in a particular geographical area.

World Health Organization recommended the following insecticides for cockroach control: Bendiocarb, Hydramethylnon, Boric acid, Fenoxycarb, Flufenoxuron, Pyriproxyfen, Hydroprene, Dinotefuran, Imidacloprid, Chlorpyrifos, Chlorpyrifos-methyl, Diazinon, Fenitrothion, Malathion, Pirimiphos-methyl, á-Cypermethrin, β-Cyfluthrin, Bifenthrin, Cyfluthrin, Cyphenothrin, D, D-trans-Cyphenothrin, Deltamethrin, Esfenvalerate, Etofenprox, λ-Cyhalothrin, Permethrin, Fipronil, Sulfluramid (WHO 2006) (32).

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