Molecular detection of synthetic pyrethroid resistance in house flies (Musca domestica L.) from poultry farms

N Sathiyamoorthy, K Senthilvel, N Rani, K Ramya, G Ponnudurai and R Velusamy

Abstract
In this study adult house flies were collected in five poultry farms from different blocks of Namakkal, Tamil Nadu. The collected flies were reared in laboratory. Pyrethroid resistance was assessed in Three to five days old F₁ generation flies by topical and residual contact methods. In this study, different concentrations of cypermethrin was used for such as 0.0002, 0.0005, 0.001, 0.002, 0.004, 0.008, 0.01 µg (a.i)/µl and 0.03, 0.06, 0.12, 0.24, 0.48 and 0.96 µg(a.i)/cm² were used for topical and residual contact assays respectively. House fly strains collected from three poultry farms, exhibited mild to moderate level of resistance in topical (RR - 9.0 to 13.5 fold) as well as in residual contact (RR - 10.0 to 17.85 fold) bioassay methods. The house fly strains from the remaining two farms showed susceptibility to cypermethrin in both bioassays (RR < 1.0). The observations of house flies resistance in bioassay methods were confirmed by PCR-RFLP method. The phylogenetic analysis and multiple sequence alignment carried out for VSSC1 allele confirmed that, there was no kdr mutation in the para-type sodium channel gene in all the five farms studied. Similarly, the phylogenetic analysis and multiple sequence alignment of CYP6D1 revealed the absence of 15 bp insert in the 5'-flanking region indicating susceptibility to synthetic pyrethroids in the three farms in which houseflies survived at higher doses.

Keywords: House flies, synthetic pyrethroid resistance, poultry farms

Introduction
House fly, Musca domestica L. (Diptera: Muscidae) is a well-known pest which usually feed and breed on decaying matter, human waste and in poultry manure. Manure accumulation under the cages coupled with prevailing temperature and humidity provide an ideal environment for the breeding and development of house flies in poultry farms. Houseflies are capable to transmit many human and animal pathogens mechanically (Forster et al., 2007) [2]. The poultry farmers generally rely on insecticides as a first choice for the control of house flies. Pyrethroids are being used widely as insecticides for housefly control in many countries (Gao et al., 2012) [3] and most frequently used insecticides for housefly control in commercial poultry farms in India. Pyrethroids are axonic excitotoxins, the toxic effects of which are mediated through preventing the closure of the voltage sensitive sodium channels in the axonal membranes, leaving the axonal membrane permanently depolarized, thereby paralyzing the organism. The present study was carried to detect prevalence of pyrethroid resistance in houseflies’ surviving in the poultry farms by bioassay and PCR-RFLP methods.

Materials and Methods
Maintenance of housefly colonies
Adult flies (>100 individuals designated as parental generations) were collected from the selected five poultry farms by sweep net and introduced into the fly breeding chamber in the laboratory. These flies were reared at 33±1°C and 60-70 per cent RH and a photoperiod of 12:12 hours light and dark until the F₁ generation produced. The composition of 100gm of larval medium– includes calf feed - 65 gm, Yeast-1gm and Water - 34 ml (Pinto and Prado, 2001). Three to five days old F₁ generation flies produced by the healthy parental population, maintained in the laboratory were used for topical and contact residual bioassays.

Bioassays
Cypermethrin topical bioassay
Topical bioassay was conducted as per the method recommended by WHO (1980) [14] and Zhang et al. (2008) [12] with minor modifications using F₁ generation of 3to5 day old house...
flies with ≥ similar size. Prior to topical application, the flies were anaesthetized by keeping in freezer for 2-3 minutes. One microliter of cypermethrin, containing the required quantity of active ingredient (a.i) in microgram (µg) diluted with acetone was applied on to the mesothoracic notum of the flies using a micropipette. The test was carried out with different doses such as 0.0002, 0.0005, 0.001, 0.002, 0.004, 0.008 and 0.01 µg (a.i.) per fly. The control group flies were treated with acetone only. Each concentration was tested in triplicates and twenty adult houseflies were used for each concentration. The insecticide treated flies were transferred to plastic container with honey and sugar coated bread and then covered with muslin cloth. Both the treated and control groups were maintained under 33±1°C and 60-70 per cent relative humidity. Data on mortality, the number of house flies that survived above the diagnostic dose were preserved in 70 per cent alcohol for further molecular studies.

**Cypermethrin residual contact bioassay**

First generation house flies (3to5 day old) were bioassayed for cypermethrin residual contact method recommended by WHO (1980) [14] and Shariffiard and Safdari (2013) [13] with minor modifications at different doses of cypermethrin viz., 0.03, 0.06, 0.12, 0.24, 0.48 and 0.96 µg (a.i)/cm². Twenty adult flies each were placed into insecticides coated bottles for one hour, and they were transferred to normal container containing honey and sugar coated bread for feeding and then covered with muslin cloth. The assay for each concentration was tested in triplicates using twenty adult houseflies along with control group in bottles coated with acetone alone. Both treated and control groups were maintained at 33±1°C and 60-70 per cent relative humidity. Data on mortality, including ataxic flies considered as dead was recorded at periodic intervals up to 24 hours post treatment in the observation sheet. The population of houseflies that survived at higher doses were preserved in 70 per cent alcohol for further studies. The observed mortalities were corrected to the control mortalities using the Abbotts formula, if any and then subjected to calculate the lethal doses (LD₅₀/LD₉₀) by standard probit analysis (Finney, 1971) using SPSS software programme. Resistance ratios (RR) were determined by dividing the LD₅₀ or LD₉₀ of field collected population by the corresponding value of susceptible strain (Keiding, 1976) [4].

**Molecular confirmation of insecticide resistance**

**Isolation of genomic DNA from housefly**

DNA extraction from housefly was carried out using DNA extraction kit (QiaAmp DNA kit-QIAGEN) as per manufacturer’s instructions. The extracted DNA samples were stored at -20°C for the further PCR assays.

**Polymerase chain reaction**

The oligonucleotide primer sequences were designed based on the sequences of genes identified from database (NCBI) to select suitable target primers for PCR amplification of voltage sensitive sodium channel (VSSC) and cytochrome P450 (CYP6D1). Primer designing was carried out with the primer design software. The selected genes were specific for DNA of corresponding insecticide resistance in housefly selected for this study.

**Table 1: Primer Sequences used to PCR amplification of VSSC and CYP6D1 genes of Musca domestica**

<table>
<thead>
<tr>
<th>Species</th>
<th>Sequences used for primer designing</th>
<th>Primer sequence (5’-3’)</th>
<th>Amplicon size</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Musca domestica</em></td>
<td>Voltage sensitive sodium channel</td>
<td>Forward: 5’-TCGCTTCAAGGACCATGAACTACCGCGCTG-3’</td>
<td>335 bp</td>
</tr>
<tr>
<td></td>
<td>(GenBank: EF 592581.1)</td>
<td>Reverse: 5’-CCGAAGTTGGACAAAAGCCTAGAAGAAGAG-3’</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cytochrome P4506D1(CYP6D1)</td>
<td>Forward: 5’-AGCTGACAAAAATTTGATCAATCGT-3</td>
<td>732 bp</td>
</tr>
<tr>
<td></td>
<td>(GenBank: AF 064795.1)</td>
<td>Reverse: 5’-CATTGGATCATTTTTCTCCTAC-3’</td>
<td></td>
</tr>
</tbody>
</table>

PCR assay was performed in thermal cycler (Nexus gradient Master cycler-Eppendorf, AG-22331, Germany). The 25 µl PCR reaction was carried out in with following mixture, Master mix-13 µl, Forward Primer (10 pmol/µl)- 1µl, Reverse Primer- (10 pmol/µl)-1µl, Template DNA-2µl, Nuclease free water-8µl. The thermal cycler conditions for each primer was standardised for optimum amplification of target product. The reaction was carried out for 35 cycles for CYP6D1 and 25 cycles for VSSC under the PCR conditions given below

**Table 2: PCR cycling conditions followed to amplify VSSC and CYP6D1 genes of Musca domestica**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Steps</th>
<th>Voltage sensitive sodium channel (VSSC) gene</th>
<th>Cytochrome-P450 (CYP6D1) gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Initial denaturation</td>
<td>94°C for 3 min</td>
<td>94°C for 3 min</td>
</tr>
<tr>
<td>2.</td>
<td>Denaturation</td>
<td>94°C for 30 sec</td>
<td>94°C for 30 sec</td>
</tr>
<tr>
<td>3.</td>
<td>Annealing</td>
<td>60°C for 30 sec</td>
<td>52°C for 30 sec</td>
</tr>
<tr>
<td>4.</td>
<td>Extension</td>
<td>72°C for 30 sec</td>
<td>72°C for 50 sec</td>
</tr>
<tr>
<td>5.</td>
<td>Final extension</td>
<td>72°C for 10 min</td>
<td>72°C for 10 min</td>
</tr>
</tbody>
</table>

**PCR-RFLP method**

Detection of resistance pattern was identified by cleavage of PCR amplicons with appropriate restriction enzymes followed by subsequent analysis of the digested PCR products by agarose gel electrophoresis. PCR-RFLP analysis was carried out to detect mutation in the genes encoding voltage sensitive sodium channel (VSSC) and cytochrome-P450 (CYP6D1) in the field housefly populations using the restriction enzymes.

**Restriction enzyme digestion**

PCR amplicons of each sample (2.5 µl) was digested with respective restriction enzymes and corresponding reaction
buffer in sufficient quantity of nuclease and protease free water in a final volume of 20 µl.

**Table 3: Conditions followed for the Restriction enzyme digestion of VSSC and CYP6D1 PCR amplicons of Musca domestica**

<table>
<thead>
<tr>
<th>Specification</th>
<th>VSSC</th>
<th>CYP6D1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplicon Size (bp)</td>
<td>335</td>
<td>732</td>
</tr>
<tr>
<td>Restriction enzyme</td>
<td>Tsp 509 I</td>
<td>Hpy 188 III</td>
</tr>
<tr>
<td>Concentration of Restr</td>
<td>2 units</td>
<td>10 units</td>
</tr>
<tr>
<td>Enzyme/µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incubation time/°C</td>
<td>3.4 h/65°C</td>
<td>1.3 h/37°C</td>
</tr>
</tbody>
</table>

Five microliter of digested samples with respective restriction enzymes were electrophoresed in 1.5 per cent agarose gel in 1 X TAE buffer containing ethidium bromide for one hour to determine mutation contributing to resistance. The gels were visualised and the images were documented in a gel documentation system (Bio-Rad Gel Doc™). Direct sequencing of PCR products and sequence analysis Sequeencing was done by automated sequencing services at Eurofins Genomics India Pvt Ltd., Bangalore, India. The nucleotide sequences were analysed using Lasergene package (M/S DNA star Inc., Madison, WI, USA) Clustal W method. The sequence was submitted in the pubmed and the accession number has been assigned (GenBank Accession Number: MW119707).

**Results and Discussion**

**Topical bioassay for pyrethroid insecticides**

Treatment of house flies with different concentrations of cypermethrin (94.3%) exhibited the vairnged mortality rate between 0 and 100 per cent. The LD$_{50}$ of cypermethrin tested against $F_1$ generation flies from different farms varied from 0.001 to 0.027 µg (a.i.)/fly and the LD$_{99}$ varied from 0.007 to 0.069 µg (a.i.)/fly. From the regression equation, the LD$_{50}$ and LD$_{99}$ value of cypermethrin were calculated and the levels of resistance are shown in Table 4.

![Table 4: LD$_{50}$, LD$_{99}$ and RR values of cypermethrin in topical bioassay](image)

*In this study, three field housefly strains showed mild (Farm-I to moderate (Farms II and V) level of resistance to cypermethrin (RR - 9.0 to 13.5 fold) when compared to susceptible populations. While the fly population from farms III and IV was found to be susceptible to cypermethrin (RR - 0.5 to 1.0). The results of the current study showing that, the existence of susceptible to moderate level resistance flies in this poultry belt. The findings are in comparable with the reports of Pap and Farkas (1994) [9], Ong et al. (2015) [10] and Kustiati et al. (2016) [5] who observed the existence of susceptible to moderate levels of resistance in the strains of house fly population to pyrethroid compounds. While, Levot and Hughes (1989) [6] reported the field populations of houseflies from Sydney showed no resistance to synthetic pyrethroid (RR < 1.0) when tested by topical bioassay procedure. On the contrary, Liu and Yue (2000) and Zhang et al. (2008) [12] noticed a very high level resistance in the field populations. The occurrence and level of resistance in an area could depend on insecticides used in that locality, frequency and duration. The resistance of house flies to insecticides vary from place to place, as they are the insect species that has shown the great ability to develop resistance to insecticide (WHO, 1991) [15].

**Residual contact bioassay for pyrethroid insecticide**

The house flies under study treated by residual contact method using different concentrations of cypermethrin (94.3%) showed the mortality rate ranging between 0 and 100 per cent. The LD$_{50}$ of cypermethrin tested against $F_1$ generation flies from selected farms varied from 0.016 to 1.268 µg (a.i)/cm$^2$ and the LD$_{99}$ varied from 0.302 to 2.761 µg (a.i)/cm$^2$. From the regression equation, the LD$_{50}$ and LD$_{99}$ value of cypermethrin were calculated and shown in Table 6. The results of current study revealed a moderate level of resistance in three field populations (RR - 10.0 to 17.85 fold) out of five but the remaining two populations remain susceptible (RR - 0.22 to 0.35) to cypermethrin. The results of this study is similar to the findings of Marcon et al. (2003) [7] who observed a moderate level of resistance in house fly populations in south-eastern Nebraska. However, very high level of resistance to pyrethroid insecticide was reported by Akiner and Caglar (2012) [1] and Sharififard and Safdari (2013) [13] in housefly populations.

![Table 5: LD$_{50}$, LD$_{99}$ and RR values of Cypemethrin in residual contact bioassay](image)

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Polymerase chain reaction
In the present study, the primers specific for two genes of *Musca domestica* were designed based on sequences selected from GenBank (Voltage sensitive sodium channel- EF592581.1; Cytochrome P450 (CYP6D1)-AF064795.1).

The amplification of 335 and 732 base pairs VSSC and CYP6D1 PCR amplicons respectively from house fly DNA was confirmed in 1.5 per cent agarose gel (Fig 1 & 2).

![PCR amplification of VSSC allele from F1 generation Musca domestica (335 bp) collected from poultry farms.](image1)

**Fig 1:** PCR amplification of VSSC allele from F1 generation *Musca domestica* (335 bp) collected from poultry farms.

![PCR amplification of CYP6D1 (Cytochrome P450) gene from F1 generation Musca domestica (732 bp) collected from poultry farms](image2)

**Fig 2:** PCR amplification of CYP6D1 (Cytochrome P450) gene from F1 generation *Musca domestica* (732 bp) collected from poultry farms.

**PCR-restriction fragment length polymorphism (PCR-RFLP)**
PCR-RFLP was chosen as the most important diagnostic assay for detecting mutation conferring resistance in the house flies in this study. The PCR-RFLP is most reliable for detecting single nucleotide polymorphism (SNP) due to the specific sequence recognized by restriction enzyme as the assay is able to give reproducible results. The PCR amplicons of VSSC and CYP6D1 gene were subjected to restriction enzyme digestion with *Tsp 509 I* and *HPY 188 III* respectively.

**Voltage sensitive sodium channel (VSSC)**
The PCR amplicons of VSSC gene from all the five farms digested with *Tsp 509 I* enzyme showed similar fragmentation pattern as shown in Fig 3. The PCR product of VSSC gene from all the farms had the fragmentation with 335, 240, 95 and 60 bp fragments indicative of susceptibility condition. Since, 170 bp fragment could not be appreciated on digestion of VSSC gene with *Tsp 509 I*, it indicates that, there was no mutation in the gene to confer resistance to pyrethroid compounds. The PCR amplicons obtained from the samples collected from house flies (F1 generation) were submitted for sequencing. The sequencing of VSSC allele revealed there was no *kdr* mutation (AATT) mutation in the L1014 F allele. Though PCR-RFLP and sequencing revealed susceptibility of the F1 generation house flies to synthetic pyrethroids, the bioassay results of farm I, II and V were contradictory to molecular assay. The partial sequence of VSSC gene from the farms was compared with ten other sequences collected from GenBank. The blast analysis showed 96.3, 95.6, 95.2 and 94 per cent homology with VSSC gene of SRS strain, CS strain and YPER strain, USA of *Musca domestica* (Fig 5 & Fig 6).
Lane 1: 100bp DNA ladder, Lane 2: Uncut PCR amplified VSSC allele from F1 generation house fly (335 bp), Lane 3-7: 335, 240, 95 and 60 bp Tsp 509 I restriction enzyme digested PCR amplified VSSC allele of F1 generation of *Musca domestica* from poultry farm I to V.

**Fig 3:** Restriction fragment length polymorphism (RFLP) of VSSC allele from F1 generation house fly from poultry farms with Tsp 509 I restriction enzyme.

Lane 1: 100bp DNA ladder, Lane 2: Uncut PCR amplified *CYP6D1* gene of *Musca domestica* (732 bp), Lane 3, 4 and 7: Restriction fragments of 732, 441 and 279bp *CYP6D1* gene from farm I, II and V respectively, Lane 5 and 6: 432 and 279 bp restriction fragments of *CYP6D1* gene of *Musca domestica* from farm III to IV.

**Fig 4:** Restriction fragment length polymorphism (RFLP) of *CYP6D1* gene of F1 generation house fly from poultry farms with *Hpy188 III* – restriction enzyme.

**Fig 5:** Phylogenetic relationship of VSSC allele of *Musca domestica* from Namakkal district, Tamil Nadu with related sequences.
Pyrethroid insecticides are used in the chemical control of house flies. The voltage-gated sodium channel (VSSC) is the main target of these insecticides, but target site insensitivity conferred by mutations in the VSSC has been a major mechanism of resistance to pyrethroids. At amino acid residue 1014, a mutation of leucine to phenylalanine (L1014 F), which is known as the kdr mutation in the para-type sodium channel gene, has been consistently associated with knockdown resistance in house flies. Resistant flies have alleles such as CYP6D1 and Vssc1 that confered resistance to permethrin and other pyrethroids.

Sequencing of the Vssc1 product of house fly which survived in the higher doses using kdr DIG long F and kdr DIG long R primers, showed that 1014 codon located three bases upstream of intron was similar to that of alleles found in the susceptible Cooper strain. The phylogenetic analysis showed that, the Vssc allele of the Musca domestica from Namakkal district formed a separate cluster and the CS strain and the New York strain evolved from this allele. Though, the F1 generation house flies survived at higher doses of pyrethroid, the results of PCR-RFLP assay revealed the absence of 170 bp fragments found in resistant population is reiterated by sequencing. The results of this study are in agreement with Rinkevich et al. (2006) (12) to identify the pyrethroid resistance in house fly population by molecular assays to study SNP/mutation in CYP6D1 and Vssc alleles. Hence, PCR-RFLP and direct sequencing can be relied on to study the mutation conferring resistance to pyrethroid compounds. Multiple sequence alignment was performed by Clustal W method in DNA star programme. The differences in the nucleotide sequences of VSSC and CYP6D1 genes of Musca domestica were compared with the sequences selected based on the BLAST algorithm from GenBank. The aligned sequences of VSSC and CYP6D1 genes are presented. The GenBank accession numbers along with origin of GenBank sequences of Musca domestica used in phylogenetic analysis are presented in Table 6.

![Fig 6: Homology and divergence between nucleotide sequence of VSSC allele of Musca domestica from Namakkal district, Tamil Nadu with related sequences.](http://www.thepharmajournal.com)

### Table 6: Sequences of Musca domestica from various geographical origins used for phylogenetic analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Strains</th>
<th>Geographical origin</th>
<th>GenBank accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP6D1</td>
<td>1. Musca domestica cytochrome P450 (CYP6D1) gene, 5' flanking sequence</td>
<td>USA</td>
<td>AF081290.1</td>
</tr>
<tr>
<td></td>
<td>2. Musca domestica cytochrome P450 6D3 (CYP6D3) gene, CYP6D3-v1 allele, complete cds; and cytochrome P450 6D1 (CYP6D1) gene, CYP6D1-v6 allele, partial cds</td>
<td>USA</td>
<td>AF200191.1</td>
</tr>
<tr>
<td></td>
<td>3. Musca domestica strain LPR cytochrome P450 6D1 (CYP6D1) gene, CYP6D1v1 allele, complete cds</td>
<td>USA</td>
<td>AF064794.1</td>
</tr>
<tr>
<td></td>
<td>4. Musca domestica strain aabys cytochrome P450 6D1 (CYP6D1) gene, CYP6D1v3 allele, complete cds</td>
<td>USA</td>
<td>AF064795.1</td>
</tr>
<tr>
<td></td>
<td>5. Musca domestica cytochrome P450 (CYP6D1) gene, CYP6D1-v5 allele, 5' flanking sequence</td>
<td>USA</td>
<td>AF081289.1</td>
</tr>
<tr>
<td>VSSC allele</td>
<td>1. Musca domestica strain A3 voltage sensitive sodium channel protein (Vssc) gene, Vssc-kdr6 allele, partial cds</td>
<td>USA</td>
<td>KM189444</td>
</tr>
<tr>
<td></td>
<td>2. Musca domestica para-type sodium alpha-subunit II6 gene, partial cds.</td>
<td>China</td>
<td>AY309437.1</td>
</tr>
<tr>
<td></td>
<td>3. Musca domestica strain YPER Vssc1 (Vssc1) gene, Vssc1-kdr allele, partial cds</td>
<td>USA</td>
<td>AY850261.2</td>
</tr>
<tr>
<td></td>
<td>4. Musca domestica Vssc1 (Vssc1) gene, Vssc1-kdr-his2 allele, partial cds</td>
<td>USA</td>
<td>AY850263.2</td>
</tr>
<tr>
<td></td>
<td>5. Musca domestica strain CS Vssc1 (Vssc1) gene, Vssc1-v7 allele, partial cds</td>
<td>USA</td>
<td>AY850264.2</td>
</tr>
<tr>
<td></td>
<td>6. Musca domestica strain Beltsville Vssc1 (Vssc1) gene, Vssc1-v8 allele, partial cds</td>
<td>USA</td>
<td>AY850265.2</td>
</tr>
<tr>
<td></td>
<td>7. Musca domestica strain CS Vssc1 (Vssc1) gene, Vssc1-v5 allele, partial cds</td>
<td>USA</td>
<td>AY850268.3</td>
</tr>
<tr>
<td></td>
<td>8. Musca domestica strain SRS Vssc1 (Vssc1) gene, Vssc1-v11 allele, partial cds</td>
<td>USA</td>
<td>AY850269.2</td>
</tr>
<tr>
<td></td>
<td>9. Musca domestica Vssc1 (Vssc1) gene, Vssc1-v6 allele, partial cds</td>
<td>USA</td>
<td>AY851288.2</td>
</tr>
</tbody>
</table>
The phylogenetic relationship of *Musca domestica* VSSC gene was derived by comparing with nine other related sequences. The VSSC gene sequence from the farms studied formed a separate cluster having close relationship with USA and China though it has showed 96.3, 95.6, 95.2 and 94 per cent homology with SRS strain, CS strain and YPER strain, USA of *Musca domestica*. This indicates, the house fly in the cluster two has evolved from the susceptible of *Musca domestica* from susceptible population. The nucleotide sequence of population under study has not undergone much change from susceptible population though the USA and China population has changed (Fig 7).

**Fig 7:** Multiple sequence alignment of VSSC allele of *Musca domestica* from Namakkal district (Tamil Nadu) with sequences from various countries (GenBank). Nucleotide differing from majority are indicated with yellow shading

**Cytochrome P450 (CYP6D1)**

The PCR amplicons of CYP6D1 gene from all the five farms digested with *Hpy 188 III* enzyme showed different fragmentation pattern as shown in Fig 4. The PCR product of CYP6D1 gene from farms I, II and V had the fragmentation with 732, 432 and 279 bp fragments indicative of heterozygous condition which could have conferred a moderate level of resistance, whereas, the house flies from
farm III and IV did not show fragmentation indicative of susceptibility to pyrethroid compounds suggesting no mutation in the CYP6D1 gene. Pyrethroids are being widely used as insecticides to control house flies in poultry farms. House flies become resistant to pyrethroids due to their prolonged use by either of the two mechanisms viz., Cytochrome P450 mediated detoxification and/or target site insensitivity (kdr). In the present study, PCR-RFLP is used to identify the presence of CYP6D1 mutation among the house fly populations. The restriction enzyme PCR amplified CYP6D1 alleles of house fly from farm I, II and V are in accordance with Rinkevich et al. (2006) indicating the occurrence of heterozygous condition (RS) conferring intermediate resistance corresponding to 279, 432 and 732 bp and farms III and IV indicating susceptible condition corresponding to 279 and 432 bp.

The PCR amplicons obtained from the samples collected from house flies (F1 generation) of all the farms were submitted for sequencing. The sequence of the farm I, II and V revealed heterozygous condition (RS) suggesting an intermediate level of resistance to synthetic pyrethroids conferred by CYP6D1 gene. The partial sequence of CYP6D1 gene from the above mentioned farms were compared with five other sequences collected from GenBank. The sequence analysis showed 85.4, 81 and 79.1 per cent homology with Cornell-R strain, OCR-strain, aabys strain and LPR strain of Musca domestica of USA respectively (Figure 8 and 9).

Fig 8: Phylogenetic relationship of CYP6D1 gene of Musca domestica from Namakkal district, Tamil Nadu with related sequences

Fig 9: Homology and divergence between nucleotide sequence of CYP6D1 gene of Musca domestica from Namakkal district, Tamil Nadu with related sequences
The phylogenetic relationship of *Musca domestica* CYP6D1 gene was derived by comparing with five other related sequences. The CYP6D1 gene sequence from the farms studied formed a completely separate cluster having no relationship with other sequences compared, though it has showed 85.4, 81 and 79.1 per cent homology with Cornell-R strain, OCR-strain, Aabys strain and LPR strain of *Musca domestica* of USA respectively (Fig 10). The sequencing and multiple alignments of the sequences of the PCR products for CYP6D1 revealed that, the PCR-RFLP method was reliable to identify the presence of CYP6D1v1 allele among the house fly population. Upon sequencing, the 15 bp insert in the 5'-flanking region defining CYP6D1v1 as reported by Qiu et al. (2007) and Rinkevich et al. (2006) could not be appreciated in the heterozygous population of house flies under study. Hence, it can be hypothesized that the fly population is a heterozygous population and still they are sensitive to synthetic pyrethroids, if they do not have any kdr mutation conferred by VSSC allele.

**Summary and Conclusion**

Treatment of house flies with different concentrations of cypermethrin bioassay, three out of five field house fly strains exhibited mild to moderate level of resistance in topical (RR - 9.0 to 13.5 fold) as well as in residual contact (RR - 10.0 to 17.85 fold) bioassay methods. The remaining two farms, house fly populations showed susceptible to cypermethrin in both bioassays (RR < 1.0). The PCR-RFLP method was carried out to detect mutation in the VSSC 1 and CYP6D1 which differentiated the susceptible population from mutant population by producing different fragmentation patterns for each gene. The interpretations of the PCR-RFLP confirmed the observations of the bioassay that intermediate level of resistance to pyrethroids in three farms. The phylogenetic analysis and multiple sequence alignment carried out for VSSC1 allele confirmed that, there was no kdr mutation in the para-type sodium channel gene in all the five farms studied. Similarly, the phylogenetic analysis and multiple sequence alignment of CYP6D1 revealed the absence of 15 bp insert in the 5'-flanking region indicating susceptibility to synthetic pyrethroids in the three farms in which houseflies survived at higher doses.
Acknowledgement

The authors acknowledge the funding provided by Tamil Nadu Veterinary and Animal Sciences University, Chennai to carry out the research work.

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