**Materials and Methods**

**(I) Study area:**

Tiruppuvanam Block, which has been selected for the present study is located in Sivaganga district, Tamil Nadu state of southern India (Plate 1). The satellite view of Tiruppuvanam is shown in Plate 2. Tiruppuvanam (Latitude N 09° 49.341’ and Longitude E 078° 15.831’), a selection grade town panchayat, is located in Sivaganga district, Tamil Nadu state of southern India. It is geographically situated at a distance of 18 Km away from Madurai on road (National Highway- NH 49) towards Rameshwaram, a pilgrimage centre. Tiruppuvanam is
located on the bank of river Vaigai. Tiruppuvanam block includes villages with a variety of geographical landscapes VIZ. plain landscapes, fertile lands and water bodies. The study area receives water for irrigation from Vaigai river, rain water stored in major and minor tanks, ground water through pump sets (Plate. 3 & 4) etc. The study area is prone to the proliferation of vectors and the incidence of vector borne diseases because of geographical locations and other ambient factors.

The study area receives rain during September (due to South West monsoon), October and November (due to North East monsoon). Rice (Oryza sativa) and sugarcane (Saccharum officinarum) are the main crops cultivated in these areas by the river water irrigation. In addition, banana (Musa paradisaica) and coconut trees (Coccus nucifera) are also planted in this area (Plate 5). Tiruppuvanam Block has different pattern of human settlements such as pucca concrete houses, thatched roof houses and huts (Plate 6 & 7). Cattle sheds and chicken roosting sites are located near the human settlements in the study area.

Tiruppuvanam Block health systems has four Primary health Centers (PHC) namely Palayanur PHC, Tiruppuvanam PHC, Konthagai PHC and Poovanthi PHC (Plate 8).
Eighteen sites in Tiruppuvanam Block have been selected for an in-depth study by stratified random sampling method and the selected study sites are shown in Plate 9. The selected sites are Puliyur, Keeladi, Elanthakulam, Manalur, Mukudi, Thavatharendal, Melarangium, Tiruppuvanam, Indira nagar, Madapuram, Enathi, Kilathari, Pethanendal, Kallorani, Thirupachethi, Muthuvanthidal, Odathur and Maranadu. The selected sites provide the real picture of geographical and demographical conditions of Tiruppuvanam Block.

(II) Study period:

A systematic survey was conducted in Tiruppuvanam Block during the period 2006 – 2008. However, a pilot study was carried out prior to the in-depth survey in the study area during 2005 - 2006. Survey has been conducted in such a way to understand the information on diversity, density, distribution, breeding and feeding behavior of the mosquito species that are prevailing in the study area. Secondary data on the incidence of vector borne diseases in Sivaganaga district and Tiruppuvanam Block were obtained for a period of four years, i.e. 2004 – 2008.

(III) Collection of mosquitoes:

To study the diversity, distribution pattern and behaviour of mosquito species in the study area, adult mosquitoes attracted to human
bait were collected from all the selected sites for a period of 24 hours continuously by the method described by Pandian and Chandrashekaran (1980). The adult female mosquitoes landed on exposed parts of the leg for biting were collected with a small transparent plastic container with cap at the time of probing on the human bait. Individual plastic container was used for each mosquito collected. Hourly collected mosquitoes were labeled and put in a separate labeled polythene bag. The mechanical aspirator and the suction tube were also used to collect resting adult mosquitoes. The collected specimens were brought to the laboratory and identified with standard identification keys (Christopher 1933; Barraud 1934 and Reuben et al. 1994). The mosquito identification was carried out by the entomologists at the Centre for Research in Medical entomology (CRME), Department of Health Research, Indian Council of Medical Research, Madurai. The identified mosquitoes were preserved at -20°C for future reference and analysis.

**Molecular characterization of mosquitoes:**

To identify the mosquitoes using molecular tools, barcoding analysis was carried out. Immature mosquitoes were collected from the selected villages in Tiruppuvanam Block. The samples were brought to the laboratory of the Post Graduate and Research Department of Zoology, The American college, Madurai and allowed to emerge into adults.
Freshly emerged adults were identified as per the standard taxonomic keys. Total genomic DNA was extracted from the whole body of individual mosquito using commercially available DNA extraction kit (Genei Pvt limited, Bangalore) as per the manufacturer’s instructions.

Polymerase chain reaction (PCR) was carried out with the partial *Cytochrome c oxidase* I (CO1) gene region specific primers as described by Simon *et al.* (1994). Two primers were used. The first primer (C1-J-1718 - 5’-GGAGGATTTGGAAATTGATTAGTTCC- 3’) was used for the amplification of the gene in the forward direction and the second primer (C1-N-2191 - 5’-CCCGGTAAAATTAAAATATAAACTTC- 3’) was used for amplification in the reverse direction.

PCR reaction was carried out as per the methodology described by Collins *et al.* (1987) with minor modifications. In 0.2ml PCR tubes, 25 µl PCR reaction master - mix was prepared and used throughout the study. The commercially available PCR mastermix supplied by Genei, Bangalore, India was used. One µl (approximately10 ng) of genomic DNA was used in each reaction. The PCR thermal cycling profile used was; one cycle of 2 min at 94$^\circ$C followed by 36 cycles of 40 sec at 94$^\circ$C, 40sec at 51$^\circ$C and 1 min at 72$^\circ$C with a final step of 5 min at 72$^\circ$C. PCR was carried out in a PTC100, thermal cycler (MJ Research, USA).
After the PCR amplification, the PCR product was visualized as per the standard protocol described by Sambrook and Russell (2001). Briefly, one part of the PCR product was mixed with 5 parts of the loading dye and loaded in the agarose gel. The amplified products were run in 2.0% agarose gel. The product images were documented with the Gel documentation system (Biorad, USA).

Though the PCR reaction amplified the amplicon for all the mosquito species studied, DNA sequencing was carried out only for the four vector mosquitoes namely, *Culex tritaeniorhynchus*, *Culex quinquefasciatus*, *Aedes aegypti* and *Aedes albopictus*. The PCR products were purified with commercially available PCR purification Kit supplied by Millipore, India and sent for commercial sequencing services to MWG, Bangalore. The products were sequenced in single direction using the same primers mentioned above.

The nucleotide sequences thus obtained was BLAST analyzed in NCBI databank. Sequence divergences among the species were studied using kimura two-parameter distance model (Kimura 1980). Phylogenetic analyses were carried out using MEGA 4.0 software (Tamura *et al.* 2007). This would help to relate the four selected vectors in the study area. The partial CO1 gene sequences of *Culex tritaeniorhynchus*, *Culex quinquefasciatus*, *Aedes aegypti* and *Aedes albopictus* has been
submitted to the Genome Data Bank. Each sequence has been allotted with an accession number for further reference.

**Diversity of mosquitoes:**

The biodiversity of species richness index (α index) was calculated by using the following formula (Southwood 1978).

\[
\alpha = 1 - \sum \left( \frac{x_1}{t} \right)^2 \left( \frac{x_2}{t} \right)^2 \left( \frac{x_3}{t} \right)^2 + \ldots \ldots \ldots \ldots \left( \frac{x_n}{t} \right)^2
\]

Where \( \alpha \) = species richness Index,

\( X \) = Number of each species in a selected site,

\( t \) = Total number of mosquitoes of all species.

If the ‘\( \alpha \)’ index is found to be closer to 1 the biodiversity species index is said to be very high.

To determine the pattern of occurrence of different species of mosquitoes in the study area, the following formula was applied (Rydzanicz and Lonc 2003).

\[
C = \frac{n}{N} \times 100
\]
Based on the score of percentage, the following distribution patterns were interpreted:

- Sporadic pattern \( (C = 0.1 - 20 \%) \)
- Infrequent pattern \( (C = 20.1 - 40 \%) \)
- Moderate pattern \( (C = 40.1 - 60 \%) \)
- Frequent pattern \( (C = 60.1 - 80 \%) \)
- Constant pattern \( (C = 80.1 - 100\%) \)

**Density of different species of mosquitoes:**

To understand the density of the mosquito fauna in the study area, adult mosquitoes were collected in the eighteen selected sites. The density pattern was calculated by using the following formula adopted by Rydzanicz and Lonc (2003).

\[
D = \frac{I}{L} \times 100
\]

- \( D \) = Density
- \( L \) = Number of all specimens
- \( I \) = Number of specimens of each mosquito
The density of the mosquito species is expressed in percentage. Based on the percentage, three patterns of density have been described namely;

- Satellite species $D = < 1\%$
- Subdominant species $D = 1 < 5\%$
- Dominant species $D = > 5\%$

**Distribution pattern of mosquito species:**

The adult mosquitoes were collected using human bait using the clean small plastic containers as per the methodology described by Pandian and Chandrashekaran (1980) in the selected sites during the study period. The occurrence of species was plotted in the study area map in their respective collection site. Then locations were connected with line and the distribution pattern was drawn. Based on the distribution, three types of distribution patterns were identified namely uniform pattern, discontinuous pattern and restricted distribution pattern.

**Blood meal analysis:**

To determine the blood feeding pattern and the host selection behavior of mosquitoes, fully blood fed and adult resting female
mosquitoes were collected from the study area. The availability of common animal hosts and their close association with human in the study area is shown in the Plate 10. The mosquitoes were collected from the resting places like, inside the human dwellings, near cattle sheds, bushes in the periphery of human dwellings etc. with the help of a mechanical aspirator during day time. The collected mosquitoes were transferred to labeled glass tubes and transported to the laboratory on wet ice. Anesthetized adults (with ether) were identified and the blood meal was squeezed individually as a thin smear on the filter paper (Whatman No.1). The blood smear on the filter paper was air-dried, stored in a sealed polythene bag and preserved at +4°C. On the filter paper, details about the place of collection, date of collection and the name of the species were marked. The blood meal of different mosquito species were identified by agarose gel diffusion technique adopted by Reuben et al. (1992). The antisera needed for the agar gel diffusion test were procured from Serological Institute of India, Ministry of Health and Family Welfare, Kolkata, India.

The agar gel diffusion (AGD) methodology adopted was as follows; serum from each host blood sample was eluted by soaking half of the finely chopped blood smear on the filter paper in 50 μl of saline. The soaked smears were incubated at +4°C over night. One percentage of agarose in barbital buffer (pH 8.6) was prepared and overlaid on clean
glass slides. After solidification, wells were carefully prepared using a metal borer with the help of a template. A total of 24 wells (in three rows) with a diameter of approximately 2mm (with a distance of 5mm between each well) were made. An aliquot of 5μl of the eluted serum was loaded in each designated antigen well with the help of a micropipette. Five microliter of host specific antiserum was added in the designated antisera well. Appropriate positive and negative controls were maintained in each slide. The slides were kept at a moist chamber and incubated at 37°C for four hours. The sera and the antisera loaded in the agar gel well move through the gel by capillary action and meet at a point. If the antiserum is specific to the antigen, a precipitin line would appear in the gel. The agar gel diffusion slide has been shown in Plate. 11. By using the following formula Anthropophilic Index (AI) and Zoophilic Index (ZI) were calculated.

\[
\text{AI or ZI} = \frac{\text{Number of blood-fed positive mosquitoes tested}}{\text{Total number of mosquitoes collected}} \times 100
\]

Based on the blood meal analysis, the anthropophilic or zoophilic feeding pattern of the mosquitoes in the study area were worked out. The result thus obtained was documented with a digital camera. The data were analyzed and the host feeding preference of the mosquito species was confirmed.
**Biting pattern of mosquitoes**

Biting rhythm of different mosquito species were studied as described by Pandian and Chandrashekarar (1980). Plotting of hourly catches will show a temporal pattern. It may be either rhythmic or arrhythmic. If it is rhythmic, it will be either diurnal or nocturnal or crepuscular. Mid-point value of biting for each mosquito species was calculated to compare the rhythmic biting pattern in the diel cycle.

**Breeding behaviour of mosquitoes:**

To study the nature and type of different breeding habitats of mosquito species in the study area, larvae and pupae were collected from all the available breeding habitats. The two types of habitats namely; on the ground (Paddy field, irrigation water canal, sugar cane field, ponds and kaccha drains) and above the ground (Leaf axils, discarded cement cistern, discarded containers, cement tank, metal drum, mud pot, grinding stone, plastic drum, coconut shell and tyre) were searched for immature stages throughout the study area by using the short - handled dipper having 15cm diameter (Reuben 1978). The larvae and pupae in the leaf axils, grinding stones, plastic drums, mud pot and tyres were collected with pipettes fitted with a rubber bulb. The different mosquitogenic conditions available in the study area were shown in Plate
12 – 14. The collected immatures were transported to the laboratory in labeled plastic containers with perforated cap and reared in plastic trays. Larvae were fed with dog biscuit and yeast extract powder. The emerged adult specimens were collected in glass tubes and identified to species level based on the morphological characteristics under Carl Zeiss stereomicroscope (Stemi 2000C, Germany) by using standard taxonomic keys and stored at -20\degree C (Christopher 1933; Barraud 1934 and Reuben et al. 1994).

**Surveillance and incidence of vector borne diseases:**

Data about the surveillance system of public health department in the study area were obtained from Tamil Nadu Public health department publications and news bulletin. The pattern, networking and personnels involved in the monitoring system were collected. The information about PHC, HSC etc. and records of the incidence of mosquito – borne diseases such as filariasis, malaria, JE, dengue and chikungunya for the period from 2004 to 2008 from the government records with the help of public health officers were obtained. Then the data were analysed to review the incidence, emergence and outbreaks of mosquito borne diseases.

The contribution of Private medical practitioners (PMP) in monitoring the health status of local public particularly due to vector
borne disease is very important. In many instances, PMP bring out the unusual emergence of a particular disease to the government public health authorities notice. The availability of easy health care facility in many places at an affordable cost and the increased awareness among the rural and suburban public encourage them to approach the private medical practitioners than the government PHC’s for medical treatment. In several instances, the private medical practitioners act as “whistle blowers” of health status of the community. Hence, an attempt has been made to undertake a survey among private medical practitioners to obtain the information about the incidence of vector borne diseases in Tiruppuvanam Block area during the study period 2006 - 2008. The interview schedule is given as Annexure- I.

Knowledge, Attitude and Practice (KAP) study:

The KAP survey was carried out in the study area. The total population of the Tiruppuvanam Block, comprising 4 Primary health Centers (PHC) was 1,07,058. The selected rural areas for the KAP study were as follows: Odathur (n-29 [390]), Thirupacheti (n-34 [1656]), Mukudi (n-29 [738]), Puliyur (n-42 [1013]), Tiruppuvanam (n-46 [1716]) and Melarangium (n-20 [1060]).
The KAP questionnaire was prepared as per the competent authorities prescriptions and with the help of a Sociologist and Medical entomologist. A copy of the questionnaire of the KAP survey is enclosed as Annexure - II. It comprises 45 questions addressed the following major categories; (i) demographic conditions, (ii) knowledge and perceptions about mosquito borne infections, (iii) knowledge about the breeding, feeding behavior of mosquitoes, (iv) attitude of the community towards mosquito elimination, control and involvement, (v) practices followed by the respondents to reduce mosquito nuisance. These questions were asked face to face method. The questionnaires were originally prepared in English language and were translated in to Tamil language in order to make the local population understand the questions. All the age groups of the population were included in the study. The interview was carried out during day time by door to door survey method. Before starting the survey, necessary permissions from the village president was obtained. Informed consents were obtained from the adult participants.

**Sampling and collection:**

The study villages (six villages) were randomly selected within the Tiruppuvanam Block. A single survey team comprising one research scholar and two assistants who carried out the survey. The team visited
the head of the village (Panchayat president). A local person’s guidance was sought in each village to visit the households. Interview was carried out in the veranda (thinnai) of the households. Then the data were processed by using appropriate software.