CHAPTER III

METHODOLOGY

3.1 Study Design

This study employed a cross-sectional design took place in Bang Rieng agricultural community, Southern Thailand. The study was designed to determine organophosphate pesticide exposure of preschool children (2 to 5 years of age) living in the community. Sample collection was carried out over two sampling periods; dry season (April-May 2004) and wet season (September-October 2004). Samples were repeatedly collected for each season.

3.2 Population Recruitment

Families within preschool children were identified through community organization including public health office and day care center. A total of 54 volunteer children was recruited based on stratified random sampling. Children living within either farm or non-farm families inside or near the vegetable farm area, were considered to be the "farm children" (n=37). Children living with non-farm families in a rubber plantation area located 7-8 km from the farm area though within the same sub-district were referred to as the "reference children" (n=17). A GIS map of the sampling location is shown in Figure 3.1. During the first visit, the study information was explained to children's parents. Parents who chose to enroll their children in the study were asked to sign a consent form and provide contact information. Parents who agreed to participate in the study had to return a completed questionnaire on the day of sample collection.

3.3 Parental Interview

Children's parents were provided with an activity diary and were asked to record their child's activities on the day before the urine sample pickup. The parents were also asked to complete a questionnaire at the time of sample collection. The questionnaire gathered general information for each child, such as age; weight and height; information regarding parental occupation; parental education; family income; household characteristics; frequency of occupational pesticide use; hygienic practices; proximity to pesticide-treated farmland; and children's activities associated with pesticide exposure. All of the sampled homes were visited a second time, 7-10 days after the first visit, in order to collect repeatedly all samples along with the corresponding activity diary.

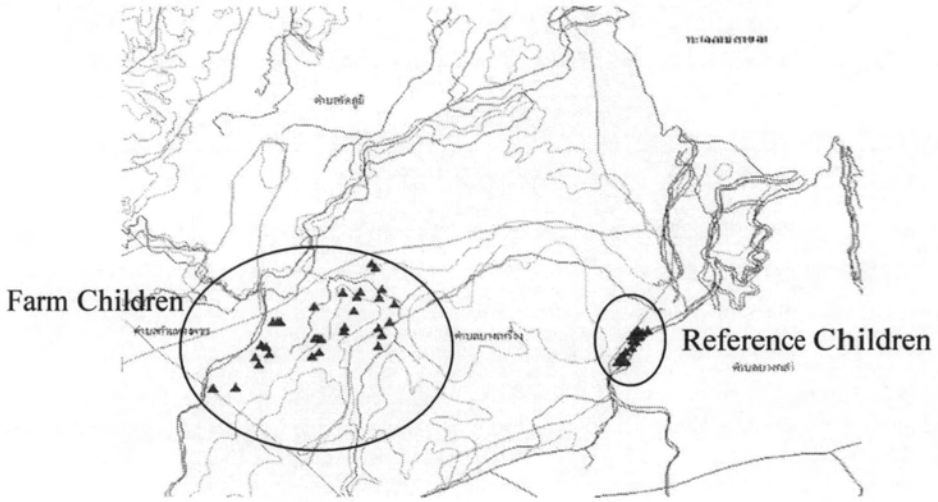


Figure 3.1 Subject's locations grouped by farm children and reference children.

3.4 Sample Collection and Analysis

3.4.1 Environmental and Personal Monitoring

3.4.1.1 Surface Soil Sampling and Analysis

Soil samples were collected from areas around the home that parents identified as common play areas of children. A composite soil sample was collected from five locations by scraping the top surface 0.5-1 cm of soil with a spatula from 26x26 template. The samples were stored at -40 °C until analysis. The method for soil sampling and analysis were performed based on the method of Simcox *et al.* (1995).

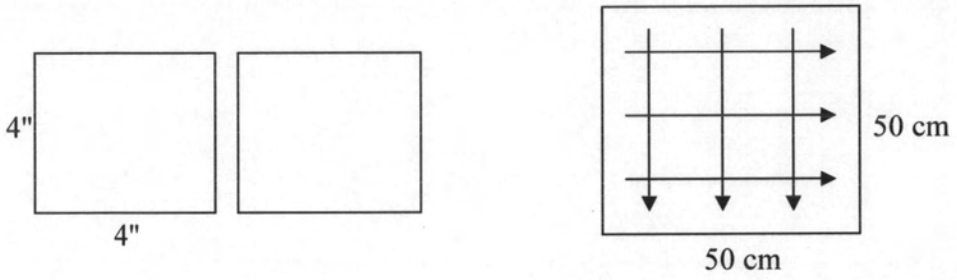
Samples were thawed to room temperature and sieved through a 425 μm stainless mesh to remove large debris. Five gram soil samples were pre-wet with 400 μL distilled water and refrigerated at 4 °C for 15-18 hr. A 20 mL aliquot of acetone was added into the soil sample and then sonicated at 20 kHz for 10 min in an ultrasonic processor. The clear supernatants were separated and evaporated to dryness under gentle nitrogen (N_2) steam. The dried samples were reconstituted with 150 μL of hexane and refrigerated until analysis.

OP pesticides in soil samples were analyzed with a Hewlett Packard (HP 6890) gas chromatography equipped with a nitrogen phosphorous detector (GC-NPD). The instrument and analytical conditions, calibration curve, and the method validation for the sample analyses are given in the Appendix B.

3.4.1.2. Surface Floordust Wipe Sampling and Analysis

Floordust was collected using surface wipe sampling technique. The surface wipe samples were collected to determine targeted OP pesticide residues in floor dust. Each dust sample was wiped from a 50 cm x 50 cm of floor using two sterile 4″x 4″ wetted gauze pads with 1-2 mL of 100% isopropanol with vertical and

horizontal three stroke wiping (Figure 3.2). Floor dust surfaces were collected from the area where children play most frequently which identified by their parents. The wipe samples were wrapped with aluminum foil, then transferred to zip-lock plastic bag and frozen at -40 °C until analysis.



(a) 4″x 4″ surgical gauze pads (b) 50 cm x 50 cm metal template

Figure 3.2 Floor surface wipe sampling technique (adapted from Lu et al, 2000)

The gauze pads were extracted by adding 30 mL of ethyl acetate and shaking on a mechanical shaker table at high speed for 30 miniutes. The clear supernatants were separated and evaporated to dryness under gentle nitrogen (N_2) steam. The dryness samples were reconstituted with 150 μL of hexane and refrigerated until analysis. The technique for wipe sample extraction was employed based on the method described by Lu *et al.* (1999; 2000).

The samples were also analyzed on HP 6890 GC-NPD. The instrument and analytical conditions, calibration curve, and the method validation for the sample analyses are given in the Appendix B.

3.4.1.3 Dermal Wipe Sampling and Analysis

Dermal wipe samples were collected using children's hand and feet wipe sampling technique. Two moistened gauze pads with 1-2 mL of 10%

isopropanol were used to wipe both hands of child, one pad per each hand. The same method was also performed on children's feet. The dermal wipe samples were wrapped with aluminum foil, then transferred to zip-lock plastic bag and frozen at -40 °C until analysis.

The extraction procedures for dermal wipe samples were performed the same as the method for extracting surface wipe samples. Dermal wipe samples were also analyzed on HP 6890 GC-NPD. The instrument and analytical conditions, calibration curve, and the method validation for the sample analyses are given in the Appendix B.

3.4.2 Biological Monitoring

3.4.2.1 Urine sampling and analysis

First morning void urine was collected on the day following the time of collection of housedust, soil, and dermal wipe samples. The parents were provided with a small polyethylene container for collecting their child's urine and were asked to transfer the urine into two labeled 15 ml screwed cap plastic tubes upon collection. The parents were also asked to place the samples in a refrigerator until it was picked up and transported on ice to the laboratory. Samples were stored at –40 °C until analysis. All urine samples were analyzed for OP metabolites and creatinine.

The metabolites were achieved by lyophilization of the urine, then the freeze dried urine samples were derivatized using pentafluorobenzyl bromide (PFBBr) and quantification by gas chromatography with flame photometric detector (GC-FPD). These procedures were developed and adapted based on the method described by Oglobline *et al.* (2001a,b) and Bravo *et al.* (2004), as described below.

a) Reagents and Chemicals

Standard reagents for DMP with 98% purity was purchased from Aldrich Chemical Co, Germany; DEP with 98% purity were obtained from ACROS Organics, NJ, USA. DMTP, DETP, and DEDTP as the potassium with 98% purity were obtained from Aldrich Chemical Co. In this study, DMDTP standard was not available in purchasing. PFBBr with 99% purity was obtained from Aldrich, USA; anhydrous potassium carbonate (K_2CO_3) was obtained from Merck, Germany. Dibutylphosphate (DBP) used as the internal standard purchased from Fluka Chemie, Switzerland.

b) Instrument Conditions

Gas chromatography used for urine analysis (HP 6890) was equipped with a flame photometric detector (GC-FPD). A capillary column, HP5 5% Phenyl Methyl Siloxane (30 m x 0.32 mm id x 0.25 μm film thickness) was used. Injection (1μL) was performed by the splitless mode. The temperature of the injector was set at 250°C. The oven temperature was programed to start at an initial temperature of 80 °C held for 2 min, then ramped to 210 °C. at a rate of 17 °C /min.

c) Sample Preparation

Lyophilization Technique

Lyophilization was performed using a Dura-Top freeze-dryer. A 5 mL aliquot of urine sample was pipetted into each screw cap glass test tube and spiked with 100 μl of 10 mg/L internal standard (DBP). The samples were then mixed and initially frozen at -40 °C. After the samples were adequately frozen, they were taken into a lyophilizing process (by Dura Top freeze-dryer) around 6-7 hours.

Derivatization Procedure

After the freeze drying process, 2 mL of acetronitrile was added to each freeze-dried urine tube, 30 mg of anhydrous potassium carbonate, 25 μL of PFBBr were added to each tube. The samples were mixed and heated at 60°C for 3 hours in a water bath. The supernatant was transferred to a clean tube and evaporated to dryness with a gentle nitrogen stream. The samples were then cooled transferred to small vials, and stored under refrigeration until analysis.

d) Standard Solution Preparation

Individual 10 mg/L stock standard solution was prepared by dissolving each of DMP, DEP, DMTP, DETP, and DEDTP in acetonitrile. The standard curves were prepared by using the spiked urine and blank urine from unexposed persons. Working standard solutions were prepared in five concentration levels of mixed DAP standards in the urine range 10-500 μg/L. A 10 mg/L of DBP was added in the solution as the internal standard, then mixed and ready for freeze drying.

The analytical results were expressed in creatinine-adjusted concentrations (microgram per gram creatinine, μg/g Cr). Urinary creatinine concentrations were also determined using the colorimetric method and expressed in unit of grams per deciliter (g/L). The individual urinary creatinine concentrations for children are presented in the Appendix C.

3.5 Data and Statistical Analysis

Data Adjustment

The distribution of the residue data was checked with the Kolomogorov-Smirnov test indicated a positive asymmetric distribution. Log transformation of data produced log-normal distribution in some but not in all groups, thus non-parametric

statistical tests were performed. The values below the limit of detection (LOD) were assigned one-half the LOD for statistical analysis (Hornung and Reed, 1990).

Statistical Analysis : using the SPSS for Windows (Version 11.5) program

- Central tendency: mean, median and percentage for describing the general information of the study population.

- Non-parametric statistical tests: use to analyze asymmetric distribution data or non-log transformed data, including the Mann-Whitney U, Wilcoxon Signed-Rank, Kruskal-Wallis, and Spearman Rank Correlation tests

Predictive Exposure Modelling

Multiple linear regression analysis was applied to evaluate the association of OP pesticide levels in environmental and personal media, including children's activity information (independent variables or predictors) with total urinary DAP metabolite levels (dependent variable). The regression models were conducted using backward and stepwise procedures. Variables with $p < 0.05$ were retained in the model; the best fitting model was selected.

The models have the same general form as follows:

$$\log(\text{concentration}) = C + \beta_1 X_1 + \beta_2 X_2 + ... + \beta_n X_n$$

In which the dependent variable log (concentration) is the natural logarithm of the total urinary DAP metabolite concentrations, β i are the regression coefficients, and the X_i independent variables; the constant C represents the background exposure level in the model.

Average Daily Dose Calculation

The average daily dose (ADD) is used to estimate the exposure for non-carcinogenic effects which calculated by the route-specific mathematical algorithms based on the following general equation.

$$ADD\ (mg/kg/day) = C \times IR \times ED / BW \times AT \qquad (3.1)$$

Where:

C = Contaminant concentration
IR = Intake rate
ED = Exposure duration
BW = Body weight
AT = Averaging time

Biologically Based Pesticide Dose Estimation

The absorbed OP pesticide doses (biologically based pesticide dose estimates) from urinary metabolite concentrations were estimated with a deterministic steady state model described by Fenske *et al.* (2000).

First Step: Total molar quantities (μmol/L) for each OP pesticide of interest were calculated by converted metabolite concentrations to their molar equivalents and summed to produce a single dialkyl phosphate for each OP pesticide, as according to the following formula.

For dicrotophos: $[\text{Dimethyl DAP}] = [\text{DMP}]/126 \qquad (3.2)$

For chlorpyrifos: $[\text{Diethyl DAP}] = [\text{DEP}]/154 + [\text{DETP}]/170 \qquad (3.3)$

For methyl parathion $[\text{Dimethyl DAP}] = [\text{DMP}]/126 + [\text{DMTP}]/142 \qquad (3.4)$

Where: the metabolite concentrations are in units of micrograms per liter (μg/L)

the molecular weight of each metabolite are in units of grams per mole (g/mol)

Second Step: The total molar concentrations were multiplied by the adjusted volume of the 24 hr urine sample and molecular weight (Mw) of parent pesticide, and are divided by the child's body weight; as according to the following formula.

$$\text{Dose } (\mu g/kg/day) = [\text{Diethyl DAP}] \text{ or } [\text{Dimethyl DAP}] \times \text{volume} \times \text{Mw pesticide} \times 1/\text{Body weight} \quad (3.5)$$

Where: Urine volume = 0.4 L/day for Asian children

Body weight = 14 kg

Molecular weight (Mw) for OP pesticide = 237 for dicrotophos ; 351 for chlorpyrifos; 277 for methyl parathion; 373 for profenofos

Non-Carcinogenic Risk Estimation

The risk estimation expressed based on the hazard index (HI). The non-carcinogenic effects were calculated according to the following relationship (US EPA., 1989):

$$\text{Hazard Quotient (HQ)} = \text{Exposure} / \text{RfD}$$

Where: Exposure = chemical exposure level, or intake (mg/kg/day)

RfD = reference dose (mg/kg/day)

Then, the hazard index is given by:

$$\text{Total Hazard Index (HI)} = \Sigma \text{ (HQ)}$$

If HI less than or equal to 1, acceptable level (no concern)

HI higher than 1, adverse non-carcinogenic effect concern