CHAPTER II

MATERIALS AND METHODS

Materials

Subjects

For prevalence study: Dogs at the Small Animal Hospital, Faculty of veterinary Medicine Chiang Mai University that older than 4 month old.

For incidence study: Dogs at the Small Animal Hospital, Faculty of Veterinary Medicine Chiang Mai University that older than 4 month old, negative with heartworm antigen test and haematocrit centrifuge technique and have not received heartworm prophylactic drug.

Equipment and supply

Haematocrit tube

Glass tube 10 mL

Clean slides

Cover glasses

Commercial heartworm antigen test kit (witness, trademark of Synbiotics Inc.,San Diego,CA,USA)

Needle

Syringe 5 mL

Microcentrifuge (Hettich zentrifugen, Mikro12-24 model)

Centrifuge (Hettich zentrifugen, EBA12 model)

Hand counter

Counting chamber

Light microscope

Auto pipette

Microcentrifuge tube

Icebox

Reagents

Ethylene diamine tetra acetic acid (EDTA)

Diff quick stain

Alcohol

10%formalin

Methylene blue

Saturated sodium chloride

0.85% sodium chloride

2% acetic acid

Distilled water

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Methods

1.Study designs

This is a cross-sectional study to determine prevalence and a longitudinal study to determine the incidence of canine dirofilariosis, using the cliental patients from the Small Animal Hospital, Chiang Mai University.

2.Prevalence study

2.1 Study design

This study is a cross-sectional study to determine prevalence, using the cliental patients from the Small Animal Hospital, Chiang Mai University. One to two ml of blood was collected by venipuncture of the cephalic vein or the saphenous vein by use of a 5-mL syringe and 21-gauge needle. The blood obtained was immediately mixed with 2.5 mg Na-EDTA. Information about risk factors of dirofilariosis was collected from dog owners using a questionnaire. Owners' information included sex and age of the dogs, their housing (outdoors or indoors) and whether the animal had participated in a heartworm prevention program. The samples were kept in 4 °C icebox during transport to hematological laboratory, faculty of veterinary medicine.

2.2 Sample size estimation

The required sample size was estimated by

$$n = \frac{1.96^2 P_{exp}(1 - P_{exp})}{d^2}$$

Where n = required sample size,

P_{exp} = expected prevalence,

d = desired absolute precision

$$n = \frac{1.96^2 (0.2471)(1-0.2471)}{(0.05)^2}$$

= 289 samples

2.3 Diagnostic method

Each blood sample was subjected to the haematocrit centrifuge technique. A haematocrit tube (MODULOHM A/S, Denmark) was filled with blood and centrifuged at 1500 rpm for 5 min. The packed cell volume was measured and the tube was cut at the buffy coat-red blood cell junction. The buffy coat smear covered by a coverslip was made according to the method of Murray (1997) and examined under the microscope using the 10x objective.

2.4 Statistical analysis

Data were entered and processed by use of the SPSS (version 8.0) and EpiInfo 2000 (version 1.1.1) programs. Associations between assumed risk factors and dirofilaria prevalence were investigated with the chi-square test option of SPSS.

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3 .Incidence study

3.1 Study design

This study is a longitudinal study to determine the incidence of canine dirofilariosis, using the cliental patients from the Small Animal Hospital, Chiang Mai University. The dogs older than 4 months, negative for dirofilariosis at the beginnig of the study and not having received prophylactic drug are included in the incidence study. The disease negative status will be established by blood examined for microfilaria (using buffy coat method) and dirofilaria antigen test (using heartworm antigen test kit, witness-tm-Merial Thailand, with at least 71% sensitivity and 94% specificity). From all dogs a blood sample of 5 mL were collected every 1-2 month for 1 year by venipuncture of the cephalic vein or the saphenous vein by use of a 5-ml syringe and 21-gauge needle. The blood samples were divided into 2 parts, 1-2 mL was immediately mixed with 2.5 mg Na-EDTA for haematocrit tube test and the other one (3-4 mL) was left to allow blood clot and serum was separated and stored in deep freezer (-20 °C) for heartworm antigen test. The animal is regarded as infected when either haematocrit or serologic test shows positive result. The newly infected dogs are recorded and the follow up of the animal stops by the positive finding month; their blood samples and feces were collected to determine eosinophilia status between heartworm infection and other intestinal parasitic infestation. The result of incidence study used to determine relative risk affected by season.

Use of likely season of infection to calculate incidence rate:

The average latent period of *D. immitis* (the time from infection to when an individual dog is infectious to others, indicated by the date of first positive demonstration of microfilaria or antigen of mature female worm in that dog) is 7 months. The likely month of infection is calculated by subtracting 7 months from the month of first positive diagnosis. The likely months of infection subsequently are categorized into average climatic seasons in Thailand, being 'summer' (March to May), 'rainy' (June to October) and 'winter' (November to February).

Calculation of incidence:

Incidence is calculated as incidence density. For each study dog (non-cases and cases) the risk period is calculated in months. For non-cases, the first month of age is taken as the begin of the period of risk, the last month of risk of in-between monthly repeated investigations is September 2001 (April 2002 – 7 months of probably latent period). The ongoing risk months until the animal get infected or the study period end up in April 2002 is counted and categorized into the 3 climatic seasons. For cases, the likely month of infection is re-calculated by use of the latent period according to above procedure. The period between the first month of age to the month of infection is taken as animal-months at risk for case dogs and the seasonal risk time of each season are determine in the same way as the non-cases.

The incidence density rate (IR) is calculated as the number of new cases over the investigation period divided by the accumulated sum of all individual's time at risk

(population-time at risk). The incidence rate is calculated for the entire investigation period and for each of the three specified seasons.

Calculation of incidence rate ratio (IRR):

The IRR is calculated as the ratio of incidence density estimates between seasons. The IRR indicates how much more likely cases occur in an exposed compared with a non-exposed population. IRR values close top 1 indicates that exposure is unlikely associated with disease frequency. The further the value from unity, the more likely it is that exposure is related to disease frequeny.

As *D. immitis* infections depend on the availability of mosquito vectors, whose abundance is primarily dependent on favorable climates, risks compared here are those due to the different climatic seasons.

3.2 Sample size estimation

Investigation so far (2000/2001) have resulted in monthly prevalence of dirofilariosis ranging between 5% and close to 20% (Pilot project). The exception is the month of March 2000 with prevalence 35%. The difference between this value and the average value of other months is about 15%. These are assumed to be new cases showing up in the early months of a year, considering seasonal exposure (rainy season) and incubation period.

Sample calculation thus follows calculation of sample sizes for prevalence, in the case of prevalence of 15%, the required sample size (n) was estimated by

$$n = \frac{Z^2_{\alpha} \times SD^2}{L^2}$$

α = 0.1

SD = $\sqrt{P(1-P)}$

L = allowable error = 0.1

P = 15%

$$n = \frac{1.645^2 \times 0.357^2}{0.1^2}$$

= 34.48

= 35 samples

3.3 Diagnostic method

3.3.1 Haematocrit capillary tube (Buffy coat method)

Each blood sample was subjected to the haematocrit centrifuge technique. A haematocrit tube (MODULOHM A/S, Denmark) was filled with and centrifuged at 1500 rpm for 5 min. The packed cell volume was measured and the tube was cut at the buffy coat red blood cell layer. A buffy coat smear covered by a coverslip was made according to the method of Murray (1997) and was examined under the microscope for the presence of microfilaria using the 10x objective.

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3.3.2 Heartworm antigen test

This was conducted using commercial heartworm antigen test kit (witness®) provided by Merial, Thailand. The procedures were as follow

Step1 : add one drop of sample (whole blood, plasma or serum) in the sample well

Step2: Follow immediately by two drops of buffer

Step3: Interpretation the result within 5 minutes (see figure5)

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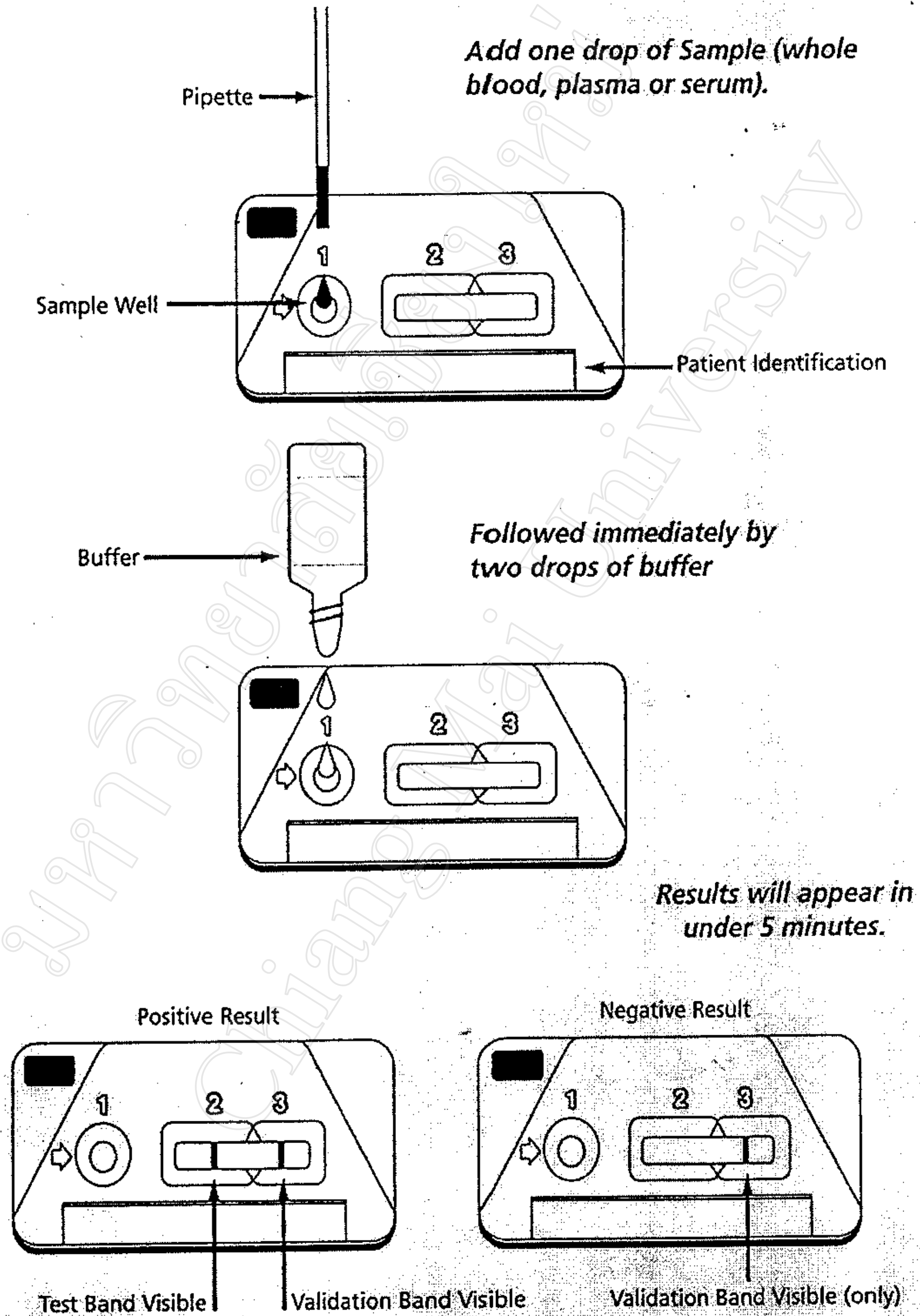

Figure3: Heartworm antigen test kit (witness®) procedure.

3.3.3 Complete blood count

Blood sample of the infected dogs were prepared for determine the complete blood count.

Procedure for differentiate white blood cells:

1. Apply a small drop of blood from a microhaematocrit capillary tube to the one end of the slide.
2. Place a spreader on the slide at an angle of 20° - 30°. Draw it back to make contact with the blood.
3. Allow the blood to run to each end of the spreader. Spread the blood along the slide in the fairly rapid but smooth motion.
4. Quickly air-dry the slide and stained with the Diff-Quick stain.
5. Examine with a microscope at a magnification of 1000x, using immersion oil.
6. White blood cells were classified as segmented neutrophil lymphocyte monocyte eosinophil and basophil.

Procedure for white blood cell count:

1. Twenty microlitter of blood were mixed with 500 microliter of 2 % acetic acid and wait for complete red blood cells hemolytic.
2. Remove the solution with pipette to fill the counting chamber.
3. Examine and count the white blood cell on the white blood cell counting square.

3.3.4 Fecal examination

For determine the eosinophilia caused by heartworm disease, the fecal examination was providing for rule out the other intestinal parasite effect. The positive dog was enema with commercial NaCl solution and the feces were kept in icebox during transportation to laboratory. Each fecal sample was subjected to flotation and sedimentation method.

3.3.4.1 Flotation method

A teaspoonful of feces is added to saturate NaCl solution to make a semisolid solution and strained the mixture through a tea strainer into a test tube. Add enough mixture or additional flotation fluid (saturated NaCl) until there is a reverse meniscus on the top of the container and then place a coverslip on the fluid drop at the top. Allow the tube to stand upright about 10 minutes, after that remove the coverslip and place it on the slide, and examine under the microscope.

3.3.4.2 Sedimentation method

Mixed a teaspoonful of feces with 50-100 mL of tap water and poured the mixture through a wire mesh screen to remove large lumps. The strained fluid caught in a beaker. The screen is rinsed with water and the debris left on the screen is discarded. The strained fluid was transferred to the petridish and left standing for 1 hour. After sedimentation, a few drops of methylene blue were added and the petridish was examined under the stereomicroscope.

3.4 Statistical analysis

Data were entered and processed by use of the SPSS (version 8.0) and EpiInfo 2000 (version 1.1.1) programs. Associations between seasonal and incidence was investigated with the chi-square test option of SPSS.

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