CHAPTER III
RESEACH METHODOLOGY

3.1 Experimental designs

The present study was designed to reveal the effects of *O. viverrini* in infected hamsters and gerbils through gross appearances using photography, histopathological changes using hematoxylin and eosin staining and trichrome staining, liver function tests (alanine transaminase, ALT; alkaline phosphatase, ALP), parasite development (body size, the maximum diameter of oral and ventral sucker, distance between suckers, number of clusters of vitelline grands, number of testes lobule, distance between testes and distance between posterior testes and excretory pore, reproductive development through eggs per worm using simple smears and eggs per gram of feces using modified formalin concentration technique, area of ovary and testes, and worm recovery during the 1-3 months post-infection. Moreover, thin-layer chromatography was used to analyze bile fluid component.

In vivo study, 48 male Syrian golden hamsters (*Mesocricetus auratus*) and 48 male gerbils (*Meriones unguiculatus*), 6 to 8 weeks old, from the Animal Unit, Faculty of Medicine, Khon Kaen University. They were divided into four groups: 1) infected hamsters (n=45; each group =15); 2) infected gerbils (n=45; each group =15); 3) uninfected (n=3; each group =1) and 4) uninfected gerbils (n=3; each group =1).

The hamsters and gerbils were infected with 50 metacercariae as assigned groups and then sacrificed on days 30, 60 and 90 of post-infection. Each animal blood, bile fluid, and liver were collected for liver function tests, bile fluid components, pathology and parasite analysis. The experimental design shows in figure 10. All protocols were approved by animal ethic committee of Khon Kaen University (AEKKU30/2551 and AE 001/54).

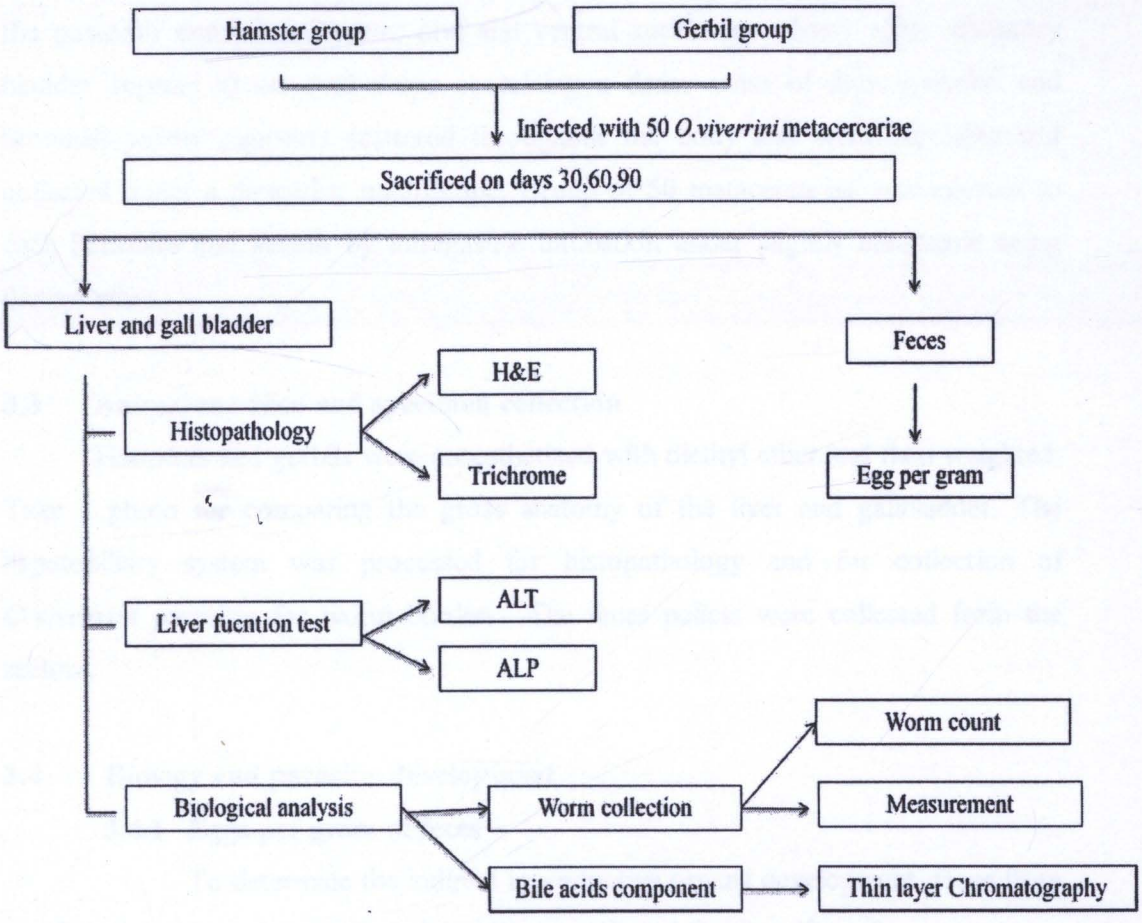

Figure 10 Experimental designs.

3.2 Preparation of metacercariae and *O. viverrini* infection in hamster and gerbil models

Metacercariae of *O.viverrini* obtained from naturally infected cyprinoid fishes such as *Puntius leilacanthus*, *Hampala dispar*, *Cyclocheilichthys apogon*, etc. The fishes were minced by electric blender in pepsin solution and then the mixture was incubated at 37°C in shaking water bath for an hour. Thereafter, the mixture was filter through a set of four sieves with 1000, 300, 250 and 106 µm apertures, respectively. The remainder was sedimented and washed several times with saline solution until the supernatant was clear. The sediment was examined for *O.viverrini* metacercariae. Finally, the *O.viverrini* metacercariae which is a double-walled cyst, oval shape, the average size of the encysted metacercaria is 201 µm×167µm and oral sucker close to

the posterior end of the worm, oral and ventral sucker are clearly seen, excretory bladder appears as an oval shape containing a dense mass of dark granules and brownish-yellow pigments scattered throughout the body and were identified and collected under a dissecting microscope. Group of 50 metacercariae was infected to each hamsters and gerbils by intragastric intubation under slightly anesthesia using diethyl ether.

3.3 Animal sacrifice and specimen collection

Hamsters and gerbils were anaesthetized with diethyl ether and then weighted. Take a photo for comparing the gross anatomy of the liver and gallbladder. The hepatobiliary system was processed for histopathology and for collection of *O.viverrini* parasites for worm burden. The feces pellets were collected from the rectum.

3.4 Biology and parasite development

3.4.1 Eggs per gram of feces

To determine the indirect reproductive organs development, feces from each infected hamster or infected gerbil was collected for detection of eggs per gram of feces with were used for finding the relationship with parasite size. Modified formalin technique was performed for the quantitative of *O. viverrini* eggs count. Two pellets feces from rectum was weighted, fixed and mixed thoroughly with 1,000 µl of 10% formalin. Then 200 µl of solution was smeared with 1% iodine solution on the glass slide and counted the number of *O. viverrini* eggs. The eggs per gram was calculated as followed.

$$\text{Eggs per gram (EPG)} = \frac{\text{Number of } O.\ viverrini \text{ eggs x } 1{,}000}{\text{Feces weight (g) x } 200}$$

3.4.2 Eggs per worm

To determine the direct reproductive organs development. Five adult worms from each infected hamsters or infected gerbils was randomized which were used for finding the relationship with parasite size. Individually *O. viverrini* crushed

with 70% ethyl alcohol by a mortar, then collected the solution into 15 ml centrifuge tube and adjusted volume with 70% ethyl alcohol to 10 ml. The solution 50 µl was smear with 1% iodine solution on a glass slide and the numbers of *O. viverrini* eggs were counted, the results were repeated three times and averaged. The number of eggs per worm was calculated as follows.

$$\text{Eggs per worm (EPW)} = \frac{\text{Number of } O.\ viverrini \text{ eggs} \times 10000}{50}$$

3.4.3 Worm recovery

To compare the worm recovery from infected hamsters or infected gerbils, worms from each liver of infected hamsters or infected gerbils were collected under saline solution.

3.4.4 Body size of *O. viverrini* adult worm

To determine the parasite development in infected hamsters or infected gerbils, 25 adult worms were randomly picked up for measurement of body size and internal organs by using the carmine staining. Adult worms were fixed in 10% buffer formalin and washed in distilled water for several times to remove formalin, stained with carmine for 60 minutes and de-stained with 1% acid-alcohol and dehydrated in serial ethyl alcohol 70%, 80%, 90%, 95% and 100% respectively for each hour. Finally, dehydrated flukes were mounted with permount solution on glass slide and measuring the body size by using the image analysis. The body width and length was measured as show in figure 11.

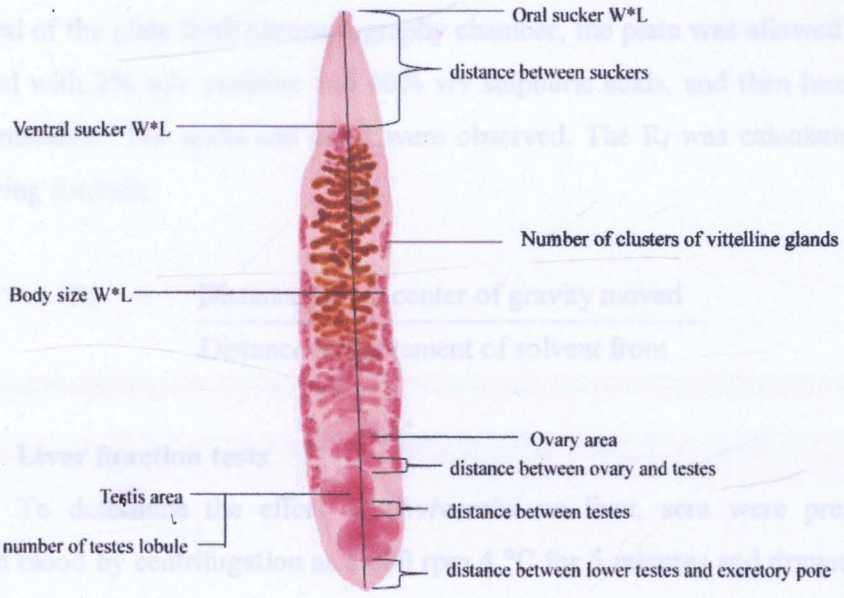

Figure 11 Measurements of distances and organs of *O.viverrini* adult worm.

3.4.5 Internal organs measurement

To determine the internal organs development of *O.viverrini* in infected hamsters or infected gerbils, the internal organs (the maximum diameter of oral and ventral sucker, distance between suckers, number of clusters of vitelline glands, number of testes lobules, area of testes, area of ovary, distance between testes and distance between posterior testes and excretory pore) of each *O.viverrini* adult were measured. The distances hold to median body line, the closely part of each organs were measurement as show in figure 11.

3.4.6 Thin-layer chromatography

To determine whether bile components affect the parasite development, bile of each hamster or gerbil was collected for TLC analysis.

A portion of 10 µl of bile fluid (sample) was mix with 10 µl methanol. Cholic acid (CA) 1 mg/ml and chenodeoxycholic acid (CDCA) 1 mg/ml in methanol were used as standards. Siliga gel 60 F254 was used as stationary phase and the mobile phase was the mixture of hexane: ethyl acetate: acetic acid: methanol; (7:23:3:2). All solution were spotted on the plate and placed in the chromatography chamber. After

removal of the plate from chromatography chamber, the plate was allowed to dry, and sprayed with 2% w/v vanilline and 10% v/v sulphuric acids, and then heat at 110 °C for 5 minutes. The spots and color were observed. The R_f was calculated using the following formula.

$$R_f = \frac{\text{Distance solute center of gravity moved}}{\text{Distance of movement of solvent front}}$$

3.5 Liver function tests

To determine the effect of *O.viverrini* on liver, sera were prepared from clotted blood by centrifugation at 3,000 rpm 4 °C for 5 minutes and drawn serum into new tube for ALT and ALP test at chemical chemistry room, community laboratory, Faculty of Associated Medical Sciences, Khon Kaen University.

3.6 Gross pathological study

To determine the gross pathological changes post *O.viverrini* infection in gerbils or hamsters. Each liver was photographed by digital camera. The liver surface, color, bile fluids, common bile duct and gallbladder were analyzed by scoring feature (Boonjaraspinyo et al., 2010).

Table 1 Gross pathological feature of gallbladder and liver criteria by macroscopic observation.

Gross pathological criteria	Score
Liver	
Liver surface	
Smooth	1
Irregular	2
Nodular	3
Liver color	
Shiny	0
Slightly yellow	1
Yellow or pale	2
Gallbladder size (mm.) (maximum width)	
Normal (3-5 mm.)	0
Mild (5-7mm.)	1
Moderate (7-9mm.)	2
Enlarge (> 9 mm.)	3
Common bile duct (color)	
Normal	0
Slightly opaque	1
Opaque	2
Bile fluid color in gallbladder	
Straw color	1
Yellow	2
Greenish	3

3.7 Histopathological study

To determine the histopathological changes post *O.viverrini* infection in hamsters or gerbils.

3.7.1 Tissue section

Liver tissues were fixed in 10% formalin and then washed by phosphate buffered saline for 12 hours (three times) and prepared for tissue section. Then the specimens were processed by the automatic machine as follow: dehydration by alcohol series; 70% ethyl alcohol for an hour (two times), 80% ethyl alcohol for an hour (two times), 95% ethyl alcohol for an hour (three times), absolute ethanol for an hour (three times) and clearing in xylene for an hour (two times) and paraffinized in the incubator at 60 °C and with paraffin solution for 1.5 hours (one time) and 2 hours (one time) respectively. Embedded tissue in paraffin block and then cut tissue section for 4 to 5 μm by microtome and coat on the slide. The tissue section slides were incubated in the incubator at 60 °C for 24 hours and kept in the room temperature.

3.7.2 Hematoxylin and eosin staining

The tissue sections were deparaffinized by xylene 3 minutes (three times) and rehydrated by series ethyl alcohol; absolute ethanol for 2 minutes (two times), 95% ethyl alcohol for 2 minutes (two times), 70% ethyl alcohol for 2 minutes (two times). The tissue sections were washed by distilled water and stained with Harris's hematoxylin 10 minutes. The tissue sections were washed in running tap water and differentiated by acid alcohol. The tissue sections were washed in running tap water for stop reaction and blued in Lithium carbonate 30 seconds. The tissue sections were washed in tap water and placed section in eosin for 3 minutes. Finally, the tissue sections were dehydrated by series ethyl alcohol; 70% ethanol for 3 minutes (three times), 95% ethyl alcohol for 3 minutes (three times), absolute ethanol for 3 minutes (three times), clearing in xylene 3 minutes (three times), and mounted slide by permount.

3.7.3 Gomori's trichrome staining

The liver section were deparaffinized in xylene and dehydration in 100%, 95%, 70% ethyl alcohol. The sections were place in Bouin's solution which

has preheated to 58 °C for 10 minutes then washed in tap water until yellow color disappear and rinsed in distilled water. Modified Weigert's iron hematoxylin for 5 minutes then washed briefly in running water and rinsed in distilled water. Placed in 0.5% hydrochloric acids in 70% ethyl alcohol for 5 seconds. Washed in running tap water for 30 seconds and rinsed in distilled water. Gomori's trichome stain for 15 to 20 minutes then quick dip in 0.5% acetic acids and rinsed in distilled water. Dehydrate and clear in graded ethyl alcohol, absolute ethyl alcohol for a minute (two times). Each slide was mounted in per mount and examined under microscope. The slides muscle fibrous were red, collagen were green and nuclei were blue to green.

3.7.4 Criteria of histological grading

The histological finding was observed under microscope.

Table 2 Histological features of gallbladder grading criteria by microscopic observation

Histopathology grading criteria	**Score**
Gallbladder	
Inflammation	
Acute	
Absent inflammatory cell	0
Focal neutrophils cells and eosinophils	1
Scattering PMN's cells and eosinophils	2
Diffuse PMN's cells and eosinophils with focal lymphocyte cells	3
Chronic	
Minimal PMN's with lymphocytes	0
Minimal PMN's and focal mononuclear cell, lymphocytes, eosinophils	1
Scattering mononuclear cell, lymphocytes, eosinophils ± plasma cell	2
Diffuse mononuclear cell, lymphocytes, eosinophils with plasma cell	3
Globlet cell metaplasia	
Absent	0
Single or discrete group	1
Two or more group	2
Diffuse	3
Epithelial dysplasia	
Absent	0
Small foci	1
Two or more foci	2
Diffuse	3
Fibrosis	
Absent	0
Mild	1
Moderate	2
Severe fibrosis with smooth muscle hypertrophy	3

(modified from Sripa et al., 2003)

Table 3 Histological feature of liver biopsy and grading criteria by microscopic observation.

Histopathology grading criteria	**Score**
Portal triad and hepatic tissue	
Portal inflammation	
Minimal/ no portal inflammation	0
Mild (sparking of inflammatory cells <1/3of portal tract	1
Moderate (increase in inflammatory cells 1/3-2/3 of portal)	2
Severe (dense packing > 2/3 of portal tract)	3
Fibrosis	
No fibrosis	0
Fibrous expansion of some portal area ± short fibrous septa	1
Fibrous expansion of most portal area ± short fibrous septa	2
Fibrous expansion of most portal area ± occasional P-P bridge	3
Fibrous expansion of portal area ± marked bridging(P-P; P-C)	4
Marked bridging(P-P; P-C)+occasional nodule (incomplete)	5
Cirrhosis	6
Hepatic bile duct size	
Normal	0
Minimal dilated	1
Moderated dilated or a parasite	2
Fully dilated or two or more parasite	3
Hepatic bile duct proliferation	
Absent	0
Focal	1
Scattering	2
Diffuse	3
Focal inflammation	
None	0
One focus or less per 10X objective	1
Two to four foci per 10X objective	2
Four to ten foci per 10X objective	3
More than ten foci per 10X objective	4

(modified from Ishak et al., 1995; Knodell et al., 1981)

3.8 Statistical analysis

The data of egg count and serum ALT, ALP levels were analyzed and presented as mean ± SEM The statistic was analyzed using One-way ANOVA (SPSS version 13.0, USA).Values were considered statistically significant when $P < 0.05$. The morphometric variables, except body length, are standardized for geometric growth prior to analysis. This correlation was achieved by dividing each measurement from particular sample by total body length of the parasite. In the case of area of ovary, testes, the raw data were divided by the whole body.

3.9 Limitation of the study

The present study was designed to reveal the opisthorchiasis in hamsters and gerbils through gross appearances using photographic, pathohistological changes using hematoxylin and eosin staining and Gomoril's trichrome staining, blood chemistry through liver function tests ALT and ALP, worm recovery and adult size, reproductive development through eggs per gram of feces using modified formalin concentration technique and biology through bile component using TLC.

3.10 Location of research conducting

The experiment was performing at the Research Laboratory, Floor 3th, Faculty of Medicine and Chemical Pharmacology Laboratory, Faculty of Pharmacology, Khon Kaen University, Khon Kaen, Thailand.

3.11 Anticipated outcome

Fundamental knowledge of this study may benefit for better understanding of *O. viverrini* infection in hamsters and gerbils. The outcomes of this study were

1. Can select the suitable host, which correlate to the purpose of study.
2. International publication.

i) "*Opisthorchis viverrini* infection cause liver and biliary cirrhosis in gerbils" submitted in parasitology research.

ii) "Comparative host parasite interaction of *Opisthorchis viverrini* infection in hamsters and gerbils through pathology and parasite development" will be submitted in parasitology research.