CHARACTERISTICS OF EPITHELIAL CELLS AND THE EXPRESSION OF CATHEPSIN L GENE IN THE DIGESTIVE TRACT OF FASCIOLA GIGANTICA

SOMPONG SRIBUREE

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE (ANATOMY)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2000

ISBN 974-663-766-5

Thesis
entitled

CHARACTERISTICS OF EPITHELIAL CELLS AND THE EXPRESSION OF CATHEPSIN L GENE IN THE DIGESTIVE TRACT OF FASCIOLA GIGANTICA

Mr. Sompong Sriburee
Candidate

Assoc.Prof. Chaitip Wanichanon, Ph.D.
Major-Advisor

Prof. Prasert Sobhon, Ph.D.
Co-advisor

Prof. Vithoon Viyanant, Ph.D.
Co-advisor

Asst.Prof. Suksiri Vichasri Grams, Dr. rer. nat
Co-advisor

Prof. Liangchai Limlomwongse, Ph.D.
Dean
Faculty of Graduate Studies

Assoc.Prof. Vijittra Lerdkamolkarn
Ph.D.
Chairman
Master of Science Program
in Anatomy, Faculty of Science

Thesis
entitled

CHARACTISTICS OF EPITHELIAL CELLS AND THE EXPRESSION OF CATHEPSIN L GENE IN THE DIGESTIVE TRACT OF FASCIOLA GIGANTICA

was submitted to the Faculty of Graduate Studies, Mahidol University for the degree of Master of Science (Anatomy)

on

March 17, 2000

Sompong Sriburee
.....
Mr. Sompong Sriburee
Candidate

.....
Asst. Suksiri Vichasri Grams,
Dr. rer. nat
Member

.....
Assoc.Prof.Chaitip Wanichanon
Ph.D.
Chairman

.....
Prof. Vithoon Viyanant, Ph.D.
Member

.....
Prof. Prasert Sobhon, Ph.D.
Member

.....
Prof. Liangchai Limlomwongse,
Ph.D
Dean
Faculty of Graduate Studies
Mahidol University

.....
Prof. Amaret Bhumiratana,
Ph.D.
Dean
Faculty of Science
Mahidol University

ACKNOWLEDGEMENT

I would like to express my sincere gratitude and appreciation to my major advisor, Assoc. Prof. Dr. Chaitip Wanichanon, for his invaluable advice, kindness, supervision and encouragement throughout my study. I am equally grateful to my co-advisor, Prof. Dr. Prasert Sobhon, for his proper guidance, advice, support, understanding, and endless patience throughout the course of this study.

I am also very grateful to Prof. Dr. Vithoon Viyanant, Asst. Prof. Dr Suksiri Vichasri Grams and Dr. Rudi Grams for their help, valuable suggestions, comments and corrections of this thesis.

I wish to thank Mr. Witoon Khawsuk, Mr. Ardool Meepool, Mrs. Pissanee Ardseungnoen, Miss. Kulathida Chaiteerayanon and the members of the Electron Microscopy Laboratory for their kindness, encouragement, and generous help that made my work possible.

I would like to thank Thailand Research Fund (Senior Research Scholar Fellowship to Prof. Dr. Prasert Sobhon) for supporting the research, University Development Commission Scholarship (UDC) and TRF for my stipend support during my study.

Finally, I would like to express my deepest gratitude to my dear parents, members in my family and my friends for their love, helpful, encouragement, and understanding during the period of my study

Sompong Sriburee

4036347 SCAN / M : MAJOR : ANATOMY ; M.Sc. (ANATOMY)
KEY WORDS :FASCIOLA GIGANTICA CATHEPSIN L / DIGESTIVE TRACT / EPITHELIUM / *IN SITU* HYBRIDYZATION

SOMPONG SRIBUREE. CHARACTERISTICS OF EPITHELIAL CELLS AND THE EXPRESSION OF CATHEPSIN L GENE IN THE DIGESTIVE TRACT OF FASCIOLA GIGANTICA. THESIS ADVISORS: CHAITIP WANICHANON, Ph.D., PRASERT SOBHON, Ph.D., SUKSIRI VICHASRI GRAMS, Dr.rer.nat., VITHOON VIYANANT, Ph.D. 133 P. ISBN 974-663-766-5

The objectives of this thesis are to classify the type of epithelium lining the digestive tract of *F. gigantica* and the expression of cathepsin L in these epithelia.

Epithelia of the digestive tracts of adults and 50-day-old *F. gigantica*, as studied by light microscopy, consist of two types: the tegumental-type which is a syncytium similar to the tegument; and the digestive-type which is composed of an array of columnar or cuboid digestive cells. The proximal part of the digestive tract, including oral sucker, buccal tube, pharynx, esophagus and part of the tract anterior to caecal bifurcation are lined by tegumental-type epithelium, whereas the caecum is lined by the digestive-type epithelium. The tegumental-type epithelium is characterized by numerous basal infoldings which extend towards the apical surface that is corrugated with cross sections of ridges or microfolds. The digestive-type epithelium is composed of columnar or cuboidal cells containing round basally-located euchromatic nuclei, prominent nucleoli, and numerous stereocilia. The digestive-type epithelial cells could be classified into 3 types: type-1, type-2 and type-3 epithelial cells, exhibit cytoplasm which are densely, moderately and lightly stained, and the quantity of zymogen granules and cell height are decreasing in that order. In contrast, the caecal epithelial cells of metacercariae are remarkably distinct from those of adult and juveniles. These cells are completely filled with large and very dense granules in comparison to those of the adult and 50-day old juveniles. Each cross section of the caecal lumen appears very irregular in shape, and surrounded by only a few epithelial cells.

The localization of cathepsin L gene (Cat L) expression in the digestive tract at each developmental stage of *F. gigantica* (metacercariae, 50-day-old juvenile and adult) was examined by *in situ* hybridization. Digoxigenin-labeled cDNA probe was produced by random priming method for the detection of mRNA coding for *F. gigantica* Cat L. Cat L mRNA was present in abundance in the cytoplasm of caecal epithelium in 50-day-old juvenile and adult stage but not detected in the nuclei. In contrast, epithelium covering the remaining parts of the digestive tract, including oral sucker, buccal tube, pharynx, esophagus and caecal bifurcation, which are tegumental-type epithelium, did not exhibit the presence of Cat L mRNA. In addition, Cat L mRNA could not be detected in metacercarial digestive epithelium.

The synthesis of cathepsin L enzyme in the digestive epithelium was investigated by indirect immunofluorescence, using mouse monoclonal antibody to cathepsin L, and polyclonal mouse anti-native *F. gigantica* Cat L and sheep anti-native *F. hepatica* Cat L as probes. In general, these antibodies gave similar result. The enzyme Cat L was localized in caecal epithelium cells in both 50-day-old juvenile and adult stage, but not in the metacercaria.

4036347 SCAN / M : สาขาวิชา: กายวิภาคศาสตร์; วท.ม. (กายวิภาคศาสตร์)

สมพงษ์ ศรีบุรี : ลักษณะเฉพาะของเซลล์เยื่อบุผิวและการแสดงออกของยีน CATHEPSIN L ในทางเดินอาหารของพยาธิใบไม้ตับ FASCIOLA GIGANTICA (CHARACTERISTICS OF EPITHELIAL CELLS AND THE EXPRESSION OF CATHEPSIN L GENE IN THE DIGESTIVE TRACT OF FASCIOLA GIGANTICA). คณะกรรมการควบคุมวิทยานิพนธ์ : ชัยทิพย์ วนิชานนท์, Ph.D., ประเสริฐ โศภน, Ph.D., สุขศิริ วิชาศรี กรามส์, Dr.rer.nat., วิฑูรย์ ไวยนันท์, Ph.D. 133 หน้า. ISBN 974-663-766-5

วัตถุประสงค์ของการศึกษา เพื่อจำแนกชนิดของเยื่อบุผิวท่อทางเดินอาหารของพยาธิ *F. gigantica* และการแสดงออกของเอนไซม์ cathepsin L ในเยื่อบุผิวเหล่านี้

การศึกษาในระดับจุลทรรศน์แสดงว่าเยื่อบุผิวท่อทางเดินอาหารของพยาธิ *F. gigantica* ตัวเต็มวัยและอายุ 50 วันมีลักษณะที่แบ่งออกได้เป็นสองชนิด ชนิดแรกคือเยื่อบุผิวที่มีลักษณะเช่นเดียวกับเยื่อที่หุ้มชั้นผิว ชนิดที่สองคือเยื่อบุชนิดลำไส้ซึ่งประกอบด้วยเซลล์ย่อยอาหารทรงแท่งหรือทรงลูกบาศก์บุภายในท่อ ท่อทางเดินอาหารส่วนต้นคือ oral sucker, buccal tube, pharynx, esophagus และท่อทางเดินอาหารก่อนที่เริ่มแตกง่ามออกเป็นลำไส้มีเยื่อบุผิวชนิดชั้นผิว ส่วนเยื่อบุผิวของซีกัมเป็นชนิดลำไส้ เยื่อบุผิวชนิดชั้นผิวมีลักษณะเด่นคือที่ฐานมันมีเยื่อหุ้มเว้าเข้าไปเป็นรอยลึก จำนวนมาก ผิวด้านบนมีลักษณะหยักไปมาและมีโครงสร้างเป็นสันคล้ายไมโครวิลไล เยื่อบุผิวชนิดลำไส้มีรูปร่างเป็นทรงแท่งไปจนถึงทรงลูกบาศก์ นิวเคลียสของเซลล์มีรูปร่างกลมและมี euchromatin มาก นิวคลีโอลัสเห็นได้เด่นชัด ส่วนยอดของเซลล์มีเสตอริโอซิเลียอยู่จำนวนมาก นอกจากนี้เซลล์เยื่อบุผิวชนิดลำไส้ยังแบ่งออกได้เป็นสามแบบตามลักษณะการติดสี จำนวนแกรนูล และความสูงของเซลล์ คือ แบบที่ 1 แบบที่ 2 และแบบที่ 3 ซึ่งไซโตปลาสมติดสีเข้มปานกลาง และจางตามลำดับเมื่อย้อมด้วยสีด่าง จำนวนแกรนูลและความสูงของเซลล์ลดลงตามลำดับ ส่วนเยื่อบุผิวลำไส้ของพยาธิระยะเมตาเซอร์คาเรียมีความแตกต่างจากพยาธิตัวเต็มวัยและอายุ 50 วัน คือภายในเซลล์มีแกรนูลกลมซึ่งมีขนาดใหญ่กว่า นอกจากนั้นหน้าตัดขวางของลำไส้มีเซลล์เยื่อบุผิวจำนวนน้อยมาก

ตำแหน่งการแสดงออกของยีนที่ควบคุมการสร้างเอนไซม์ cathepsin L ในเซลล์เยื่อบุท่อทางเดินอาหารของพยาธิ *F. gigantica* ในระยะตัวเต็มวัย อายุ 50 วันและเมตาเซอร์คาเรีย ถูกแสดงโดยวิธี *in situ* hybridization เราได้สังเคราะห์ตัวตรวจดีเอ็นเอซึ่งติดฉลากด้วย digoxigenin โดยวิธี random priming method เพื่อใช้หาตำแหน่งการกระจายของ mRNA ซึ่งเป็นตัวนำรหัสไปแปลเป็นเอนไซม์ Cat L ของพยาธิ *F. gigantica* จากการศึกษาเราได้พบการกระจาย mRNA ของ Cat L ในไซโตปลาสมเซลล์เยื่อบุผิวของซีกัม ส่วนในเยื่อบุผิวของท่อทางเดินอาหารส่วน oral sucker, pharynx และส่วนก่อนที่แตกเป็นง่ามออกเป็นลำไส้ซึ่งมีเยื่อบุผิวชนิดชั้นผิว และในเมตาเซอร์คาเรียไม่พบ mRNA ของเอนไซม์ Cat L

เราได้ศึกษาการสังเคราะห์เอนไซม์ Cat L ในเยื่อบุผิวของท่อย่อยอาหารโดยวิธีอิมมูโนฟลูออเรสเซนต์ โดยใช้โมโนโคลนัลแอนติบอดีต่อเอนไซม์ Cat L โพลีโคลนัลแอนติบอดี 2 ชนิดคือ mouse anti-Cat L *F. gigantica* และ sheep anti-Cat L *F. hepatica* แอนติบอดีทั้งสองประเภทให้ผลคล้ายกันกล่าวคือในพยาธิระยะตัวเต็มวัยและอายุ 50 วัน มีการกระจายตัวของเอนไซม์ Cat L ในเซลล์เยื่อบุผิวของซีกัม แต่ในระยะเมตาเซอร์คาเรียซีกัมย้อมไม่ติดสารเรืองแสง แสดงว่าไม่มีเอนไซม์ชนิดนี้

CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

Cat L	=	cathepsin L
Cat B	=	cathepsin B
FABP	=	fatty acid binding protein
GST	=	glutathione S-transferase
RT-PCR	=	reverse transcriptase polymerase chain reaction
ES	=	excretory / secretory
IgG	=	immunoglobulin G
FITC	=	fluorescein isothiocyanate
PBS	=	phosphate buffer saline
BSA	=	bovine serum albumin
DEPC	=	diethylpyrocarbonate
PoAb	=	polyclonal antibody
MoAb	=	monoclonal antibody
NEJ	=	newly excysted juvenile
cDNA	=	complementary deoxyribonucleic acid
DNA	=	deoxyribonucleic acid
mRNA	=	messenger ribonucleic acid
DIG	=	digoxigenin

kDa	=	kilo dalton
M	=	molar
mM	=	millimolar
rpm	=	round per minute
g	=	force of gravity
cm	=	centimeter
mg	=	milligram
ml	=	milliliter
µl	=	microliter
nm	=	nanometer
°C	=	degree celsius
RT	=	room temperature
Ca	=	caecum
Te	=	tegument
Ti	=	testis
Bl	=	bladder
Ut	=	uterus
Mg	=	Mehlis' gland

CHAPTER I

INTRODUCTION

Fasciolosis is a parasitic disease caused by the infection with the parasites *Fasciola gigantica* and *Fasciola hepatica*. *F. gigantica* is prevalent in the tropical region, while *F. hepatica* is prevalent in the temperate zone (1). Fasiolosis causes significant economic loss estimated at US$2000 millions per annum in rural agriculture communities as well as in the commercial farm sector worldwide (2). In Thailand alone such economic loss amounts to 350-400 million Baths per year (3). Accurate assessment of the economic loss from the infection with fasciola is hampered by several factors such as incomplete information on the extent of loss of meat, milk and fibre production, this is due to mortality, decreased fecundity reproduction, feed conversion efficiency and appetite loss as adversely affected by the infection. (4). In many parts of the tropic fasciolosis is considered to be the single most important helminth infection of cattle, with prevalence rate of 30-90% in Africa, up to 85% in North-East Thailand and 25-90% in Indonesia (5,6,7,8). *F. gigantica* infection in human has been reported in Africa, Egypt and Thailand (4,9,10), and increasingly recognized as causing significant human disease, with 2.8 million people infected and further 180 million at risk (11).

Many methods for controlling fasciolosis such as strategic anthelmintic treatment, grazing management, application of molluscicide, and fencing off or draining swampy areas have been used (4). However, their relevance in controlling

fasciolosis depends on economic and educational constraint since most subsistent farmers in prevalent areas lack information about the benefits of implementing such measures for controls.

Up to now the most widely used control measure for *F. gigantica* and *F. hepatica* are based on chemotherapy; however, whereas anthelmintics are commonly the main-stay programs of control for *F. hepatica*, it has not yet been used widely against *F. gigantica*. Since most farmers in the tropical region are relatively poor and can hardly afford the drugs by themselves. The common drugs that are used for control fasciolosis include Albendazole, Clorsulon, Hexachlorphene, Triclabendazole (4, 12). But long-term use of these drugs, such as triclabendazole does not prevent reinfection, and hosts in endemic area must be continuously treated. Furthermore, there is an evidence of the drug-resistant strain arising in the prevalent areas subjected to intense chemotherapy (16). Ideally, the controlling measure for fasciolosis should be low cost, readily available and applicable with little disruption to existing agricultural practice. Vaccine is deemed to be an ideal control method for long-term, low cost, and wide spread application.

Recent developments on the knowledge of the immunobiology of *Fasciola* infection in ruminants have provided new insights into the nature of relationship between liver flukes and their mammalian hosts. Molecular studies aimed at developing defined vaccines for controlling fluke infection have raised the realistic possibility that immunological control of Fasciola infection is a commercially achievable goal. Controlling fasciolosis by vaccination rather than chemotherapy will be a cheaper, more efficient, and reliable long-term solution. Before 1987, attempts to vaccinate grazing animals against *F. hepatica* had generally used crude somatic

parasite extract, mixtures of secreted parasite proteinase or radiated attenuated young parasites, such as metacercariae (14). Since then, there have been several reports of the identification and characterization of proteins from *F. hepatica*, some of which have been tested in vaccine trials with positive results. Similarly vaccination of ruminants against *F. gigantica* has been obtained using irradiated metacercaria (14), and rats have been vaccinated with extracts of metacercariae of *F. gigantica* (15). Four purified antigens from *F. gigantica*, including fatty acid binding protein (FABP), glutathione S-transferases (GST), cathepsin L (Cat L), and paramyosin have been tested as vaccine in Brahman-cross cattle in Indonesia with some positive results (16).

Cysteine proteinase, a proteolytic enzyme, is named for the thiol-containing cysteine side chain. Cysteine protinases are expressed in numerous species of plants and animals from protozoa through to mammals. There were several cysteine proteinases, isotypes; and the cathepsins is the family of related cysteine proteinases which differ in their specificities and molecular weights. Their subtypes includes cathepsin B, L, H, T, K, S (17). Cathepsins B, H and L are the most typical and well characterized proteiases in lysosomes, and they are widely distributed in many organs, most notably in gastric and duodenal tissues (18,19,20,21). Parasites tend to express several cysteine protees, which are used in breaking down host tissue for nutrition (22,23). *Schistosome* cathepsin L proteinase may be involved in the digestion of hemoglobin obtained from host erythrocyte (24). Newly excysted and juvenile parasites may elaborate cysteine proteinases to enhance their burrowing and invading into the host tissues, in order to migrate to the definite organs where they finally reside, and possibly also using these enzymes to disrupt immune function of the hosts (25). The disruption of immune attack on the exposed parasite tegument or tissues

may be performed by secreting proteinase to cover the tegumental surface (26), or conversely, rapid turnover and degradation of the parasite tegumental coating layer by its own secreted proteinases would hinder recognition by the host immune system (27).

Wijffels and coworkers (1994) vaccinated sheep with purified *F. hepatica* cathepsin L (FhCat L), in Freund's complete adjuvant, and found that rather than a reduction in worm numbers, faecal egg counts were significantly reduced (28). Dalton has also demonstrated that vaccination with two different FhCat L proteins (also termed Cat L1 and Cat L2) in Freund's adjuvant can reduce both worm burdens (up to 69%) and egg production, and viability (up to 65%) after challenge in Bhraman cattle (29). Cat L from *F. gigantica* has also been tested as a candidate vaccines in cattle, using diethylaminoethyl (DEAE) in Squelen Montani 80 as adjuvant (16). However, despite the induction of high antibody titres to Cat L, no reduction in worm burdens or faecal egg counts was observed.

In immonolocalization studies, it was found that upon using antisera generated to native cathepsin L, the antigen was localized in the gut epithelial cells of adult *F. hepatica* (30) These findings are supported by previous studies that revealed localization of cysteine proteinases in the intestinal epithelium of several other nematode and trematode parasites (22, 31, 32). Thus, Cat L appears to be proteinase that is distributed in a wide range of parasites. However, in *F. gigantica* there have not yet been similar studies of immunolocalization of CatL.

Digestive system of *Fasciola gigantica* consists of oral sucker, buccal tube, pharynx and caeca. The oral sucker is located at the anterior tip of the body and is

connected with the pharynx by the buccal tube. The caecum is continuous with the esophagus and bifurcated into left and right main caeca extending towards the posterior end of the body. Both main caeca give off numerous medial and lateral branches The epithelium of the caeca varies from tall columnar to low cuboidal with apical branching and long microvilli depending on volume of the food in their lumen (33). The ultrastructural studies in *F. hepatica* have revealed the presence of lamellar process extending into lumen of caeca, the evidence which indicates that the gastrodermis cells may be both secretory and absorptive in nature (34) In contrast, similar histological studies have not yet done in *F. gigantica.* Caecal bifurcation and caeca are the continuation of pharynx. The latter has epithelium which is continuous with the tegument layer, and in theory it should be lined by tegumental type epithelium. It is interesting to ask whether pharynx is indeed lined by the epithelium similar to the tegument or by digestive-type epithelium. And exactly where the transition from one type to the other begin. Furthermore, it could be asked whether CatL gene is expressed strictly in digestive epithelium only and begins at what point in the digestive tract.

Hence, in this study, the experiments are designed to study the histology of the digestive tract which could be correlated with the localization and distribution of Cathepsin L in *F. gigantica* at different developmental stages (metacercaria, juvenile and adult), so that the basic knowledge gained could be applied in the development of immunodiagnosis tool and candidate vaccine for *F. gigantica* infection.

CHAPTER II

OBJECTIVES

The objectives of this study are as follows:

1. To identify and study histology of the digestive system of developmental (metacercaria and juvenile) and adult stages of *Fasciola gigantica* by using light microscopy Special emphasis is given on identifying the type of epithelial lining of various parts and the transition point of tegumental type to digestive type epithelium.

2. To study the expression of Cathepsin L gene in the epithelial cells of the digestive tract and other tissues of developmental (metacercaria and juvenile) and adult stages of *F. gigantica* by *in situ* hybridization technique, using DNA probe for detection of mRNA coding for Cathepsin L.

3. To study the distribution of Cathepsin L in the digestive tract of developmental (metacercaria and juvenile) and adult stages of *F. gigantica* by using monoclonal antibody and polyclonal antibody and indirect immunofluorescence technique.

CHAPTER III

LITERATURE REVIEW

1. Life Cycle of *Fasciola gigantica*

Fasciola gigantica is a digenetic trematode, belonging to the family Fasciolidae, order Echinostomatida, super-order Anepithliocystida, subclass Digenea, class Trematoda in phylum Platyhelminthes (35). Cobbold originated the scientific name of *F.gigantica* in 1856. *Fasciola gigantica* is a trematode resembling *Fasciola hepatica*, but it is larger in size. It is a common liver fluke of the domestic and wild ruminants in the tropical parts of Asia, Africa and a few other areas. *F. gigantica* is the liver fluke whose life cycle involves two hosts: the intermediate snail and the definitive mammalian host The life cycle of this fluke is very similar to that of *F. hepatica* and requires 5-6 months for completion (Figure 1) (35). The infection occurs when the animal ingests vegetation contaminated with metacercarial cysts. The metacercaria is the infectious stage, and shows little morphological difference from the cercariae (36). Metacercaria of *F. hepatica* is surrounded by a four-layer protective cyst: 1) an outer tanned protein layer with irregular meshwork containing cigar-shaped bodies, 2) a fine fibrous layer, 3) an inner layer, consisting of mostly mucopolysaccharide, and 4) a dense compact layer composed of numerous protein sheet (37). The survival of encysted larvae depends on environmental factors, especially relative humidity and temperature. It was found that metacercariae of *F. gigantica* could survive at high

temperature and they were resistant to freezing at -2 to -10 °C, but their infectivity was decreased at -20 °C (35). After ingestion by the definitive host, metacercariae is excysted in the small intestine. Excystment in fasciola is an active process, occuring in two stages: activation and emergence (38,39). Activation is initiated by high concentrations of carbon dioxide. The emergence phase is triggered by bile. The metacercaria excysts through a small cirrcular hole in the ventral side of the cyst, and it is possible that the organism releases a proteolytic enzyme, which digest the wall of the cyst at this point In ruminants, the activation takes place in the lumen and emergence takes place in the small intestine below the opening of the bile duct, whereas in non-ruminant host activation occurs in the stomach or small intestine (40). An excysted metacercaria or newly excysted juvenile (NEJ) burrows through the intestinal wall and reach the abdominal cavity within 24 hours. The juvenile fluke move through the peritoneal cavity and liver parenchyma, and eventully reach the bile duct where they stay permanently. The adult worm burrows through the liver and creates tunnels behind itself into which it deposits its excretion and fertilized eggs. The eggs leave the liver through the common bile duct, enter the small intestine, and pass into in the fecae. The embryonated eggs deposit in fresh water for the life cycle to continue. The eggs of *F. gigantica* are large, ovoid and light yellowish-brown in color and operculated, measuring about 140x75 μm (41). Under favorable environmental conditions (water and temperature), the embryo develops in to a ciliated larval form, the miracidium, which hatches and swims about with aid of its cilia. The miracidium finds its way to a suitable intermediate host that is the fresh water snails of the genus Radix (41). It penetrates the snail' s body wall, shedding its ciliated cells, and transforms to saclike sporocyst. As the sporocyst grows, new larval forms

develop within it. These forms called rediae, leave the sporocyst and typically move to hepatopancreas of the snail host. As the primary redia grow, new daughter rediae develop within them. Eventually, rediae give rise to another embryonic type, the cercaria This form has a tail, and after leaving the redia, it eventually escapes from the snail into the surrounding water. In another of minutes, the cercaria encysts on a blade of grass or other vegetables and become metacercariae.

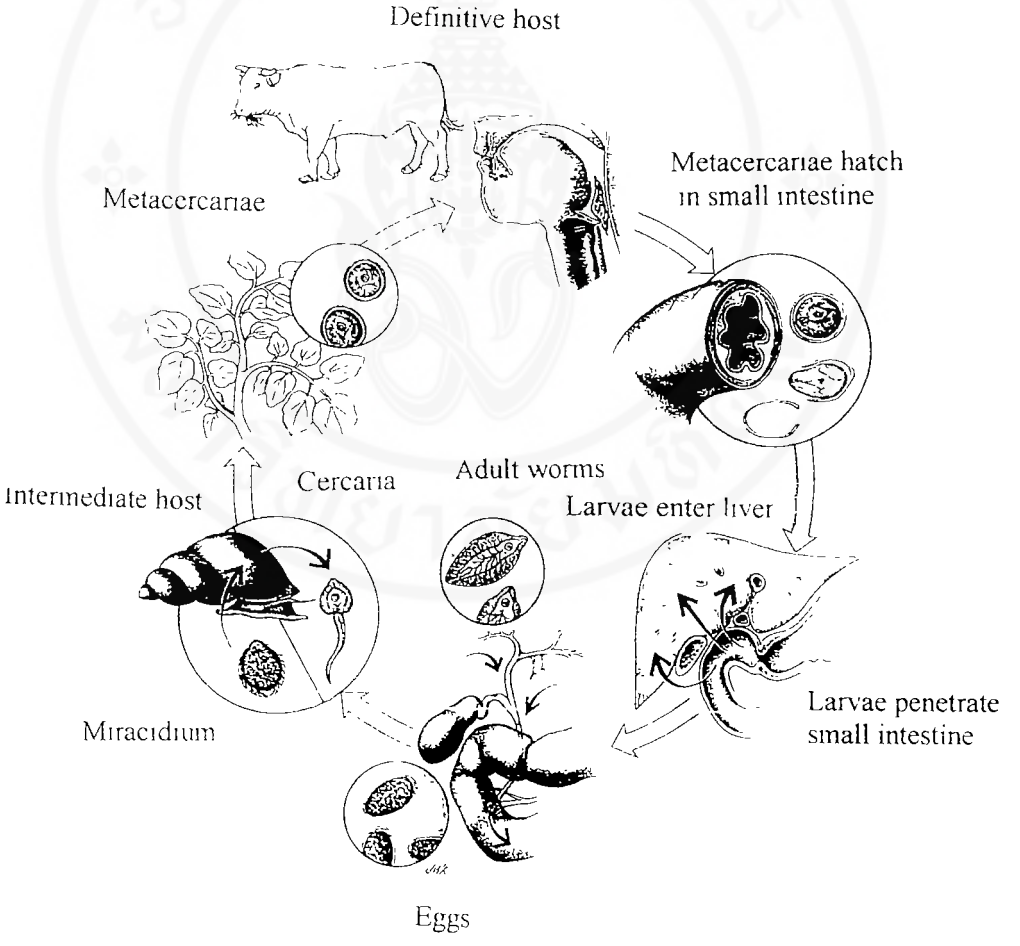


Figure 1. The life cycle of *Fasciola gigantica*
(Modified from Despommier D.D., Gwadz R.W., and Hotez P.J. Parasitic diseases. 3rd ed. New York: Springer-Verlag, 1995)

2. Development of the Digestive Tract of Trematodes

Trematode parasites live in a wide variety of habitats, may be in direct contact with host tissue, or are bathed in host fluids. The digestive tract is the region that can be regarded as representing the host-parasite interface of the trematode. The source of food for parasite is the host substance and tissue. This host material is broken down extracorporeally by parasite enzymes prior to being taken in through the mouth. The method of feeding is essentially suctorial, in which food is sucked through the mouth into the gut and, because of the absence of an anus, the gut contents are retrogradely transported to the oral sucker.

2.1 Digestive Tract of the Larval Stage

2.1.1 Redial Digestive Tract

The digestive system of the redial stage possesses a simple gut consisting of mouth, oral sucker, a short esophagus and a simple, undivided caecum which are short or extend through the length of the body. The epithelium of the oral sucker and esophagus of the redia of *Parochis acanthus* (42) and *Fasciola hepatica* (43) is lined by a continuation of the external tegument. In both of these species, secretory gland cells that produce electron-dense, membrane-bound vesicles surround the esophagus The composition of the secretion in esophageal glands in *P. acanthus* contains carbohydrate and protein (42). There is the transition from the esophagus to the caecum. The caecum is lined by a flattened epithelium in the case of *Neophasis lagenniformis* (44). The epithelium has long septate desmosomes linking adjacent cells. The cytoplasm is dense and contains a nucleus, rough endoplasmic reticulum

(RER), free ribosomes, Golgi complexes and particles of alpha glycogen. The basal membrane of these cells forms tubular invaginations that project into the cell cytoplasm. The most prominent feature of the caecal epithelium is the presence of projections up to 2 μm long, which arise from the plasma membrane (44).

2.1.2 Cercarial Digestive Tract

The digestive system of cercaria consists of a mouth surrounded by an oral sucker, a prepharynx, and a muscular pharynx followed by esophagus. There are two caeca which vary considerably in size in different species. The ultrastructures of the cercarial gut of *Schistosoma mansoni* , *Zoogonoidaes vivaparus* and *Neophasis lagenformis* were studied (44). The oral sucker, prepharynx and esophagus are lined by a syncytial tegument that cover the outer surface of the parasite. There is an abrupt contrast between the termination of the tegument lining the anterior part of the gut and the proximal part of caecal gastrodermis. The cercarial caecal epithelium resembles very closely the gastrodermis of rediae. The gastrodermis is cellular with lateral septate desmosomes and with the luminal surface thrown into long lamellae 25 nm thick and 1-3 μm long The intracellular spaces may be distended and contain numerous small vesicles. The basal plasma membrane forms tubular invagination up to 0.5 μm deep (44). The gastrodermal cells of *N. lagenformis* are rich in RER, Golgi complexes, mitochondria, lipid droplets, alpha glycogen particles and numerous membrane-bound vesicles (44)

2.1.3 Newly Excysted Juvenile Digestive Tract

The gross development of the gut during the growth of *F. hepatica* in the mouse has been described by Dawes (1962). Initially in the NEJ, the caeca are short and show signs of elongation in the first day following excystment. Lateral branches begin to develop in day 3 post-infection, following invasion of the liver. By day 8 post-infection, there are 13 club-shaped branches on each side of the body and they are club shaped. Secondary and tertiary branches are present by day 11 post-infection, the caecum displaying much of the complexity of the fully develop system (45). In the NEJ, the caecal lumen is irregular in shape and is enclosed by relatively few cells. The cytoplasm of these cells contains mitochondria, glycogen and extensive RER with narrow cisternae. These cell is possess of numerous large, dense secretory granule produced and stored during the metacercarial stage. The number of secretory granules is reduced after post excystment, leading to the suggestion that they contain hydrolytic enzymes for use in excystment, penetration through the gut wall, migration and penetration to the liver capsule (46). After 3 days post infection juvenile were penetrated to the liver capsule and recovered from the hepatic parenchyma. The caecal cells start to assume a more adult-like morphology (46). The large granules were lost and the Golgi complex produced a smaller type of secretory granule.

2.2 Digestive Tract of the Adult Trematodes

The digestive tract of the adult trematode can be divided into two distinct regions: 1) the foregut, consisting mouth, pharynx and esophagus, and 2) the paired caeca, which end blindly and their lateral branches are highly branched. Most of observations on the morphology of the digestive tract are confined to caecum. The

digenean caeca appears to be syncytial in most species with the quite definite exception of *Fasciola hepatica* in which the layer is truly cellular (44). The epithelial lining of caeca consists of a continuous single layer of cells of one basic type, although they show considerable variation in the fine structure (36). The differences in structure reflect different functional states, the cell undergoing cyclical transformations between absorptive and secretory phases. Early studies at the light microscope level demonstrated that the cells of the gastrodermis exhibited a secretory cycle which was associated with the transformation of tall columnar cell into the short cells (47). The gastrodermis must also have an absorptive function and the exact relationship between both these functions. Robinson and Threadgold (1975) suggested that the variation in the morphology of the gastrodermal cells represented a continuing variation of a single cell (34). They established three major categories, group-I, II and III Cell in the secretory phase (group-I) contained the tall columnar cells with many dense secretory granules, abundant and active Golgi complexes, an extensive network of RER cisternae and numerous mitochondria. The absorptive cells (group-II) are low columnar with more numerous apical lamellae, between which lie exocytosed secretory granules and membranous whorls. They possess few and largely inactive Golgi complexes, show a lack of secretory granules and contain numerous cytoplasmic bodies. The group-III cells are cuboidal and 10-20 μm tall. The lamellae of these cells are short and both Golgi complexes and secretory granules are present. The function of these cells is not clear. Robinson and Threadgold (1975) suggested that they may be concerned primarily with the movement of materials in the lumen of the gut (34).

In contrast to the caecum, the anterior region of the gut comprising oral sucker, pharynx and esophagus has not been studied intensively. The most detail accounts to the gut of *Schistosoma mansoni* (48) and *Schistosomatium douthitti* (49). The oral sucker is lined by tegument which in most details resembles that covering the general body surface. Within the pharynx, the tegument becomes deeply fenestrated with long narrow folds of tegument. The folds are larger and narrower in the esophagus and contain extensions of the basal plasma membrane. The anterior esophagus has a lining resembling tegument, but the posterior esophagus possess additional distinctive features in that it contains characteristic secretory bodies within the tegumental folds. The secretory bodies are electron-dense rod shaped and rounded granules produced by the esophageal gland. In *Megalodiscus temperatus* the mouth, pharyngeal pouches and esophagus are covered by tegument (50). In *Schistosoma* and *Megalodiscus* the transition from tegumentory surface to gastrodermis is abruptly marked by a long junctional complex (48,50).

3. Proteinases of Fasciolidae

Proteinases are enzymes that catalyze the hydrolysis of peptide bonds. Proteinase that degrade from the end of a polypeptide chain are called peptidases or exopeptidases, while those that catalyze cleavage of an internal peptide bond are called endopeptidases. Proteinases are separated into four major classes based on their active site amino acid residues. serine, cysteine, metallo-, and aspartic proteinases (51)

Many parasites used proteinases for their living, including tissue penetration, digestion of host tissue for nutrition and evasion of host immune responses. Proteinases of all the four major groups have been characterized in parasitic helminth, but the largest number reported belong to papain superfamily of cysteine proteinase (52). Cysteine proteinase contains an active site cysteine with its thiol side chain functioning in substrate binding. Catalysis by cysteine proteinases are also facilitated by two other active sites histidine and asparagine (51). Papain superfamily can be divided into two major branches based on gene sequence relationship. Branch A contains the cathepsin Bs, cathepsin Cs and cathepsin Xs, while branch B contains the cruzipains, cathepsin L, papain-like and aleurain/ cathepsin H-like proteinases (52).

Fasciola contains proteolytic enzyme in both the excretory-secretory (ES) material, which can be collected from the adult parasite *in vitro*, and in somatic extracts (53). A recent analysis of the ES products secreted by *F. hepatica* adults revealed the presence of several proteolytic enzymes of the cysteine proteinase class. One of these proteins has been purified and characterized as a cathepsin L like enzyme (30,25). Chapman and Mitchell (1998) have demonstrated that the secreted cysteine proteinases released by *F. hepatica* are predominantly of molecular weight 27-28 kDa (25) Biochemical studies from a number of laboratories have shown that proteinases secreted by the adult *F. hepatica* are related to the mammalian cathepsin L (28,30,54,55,56). Although two-dimensional electrophoresis of ES products demonstrated that the proteinases are highly heterogenous , two major forms of 27 kDa and 29 5 kDa have been purified and characterized, and were termed cathepsin L1 and cathepsin L2 respectively (28,30,56). The two enzymes have distinct substrate specificities against fluorogenic peptide substrates.

Spithill, *et al.* (1991) has demonstrated that the occurrence of cysteine proteinase in the ES product of adult *F. gigantica* which migrate as a triplet bands of molecular weight 27-28 kDa, and shown that these proteinases cross-react with antiserum raised to *F. hepatica* secreted proteinases (55) Fagbemi and Hillyer (1991) characterized the proteolytic enzymes from extract of adult worms of tropical liver fluke, *F. gigantica.* These enzymes were cysteine proteinases that ranged in molecular sizes from 26 to 193 kDa as revealed by gelatin-substrate PAGE. The most predominant proteinase, was partially purified and shown to be capable of degrading immunoglobulin (58). These enzymes are most likely the homologues of the *F. hepatica* cathepsin L proteinases (59).

Screening of adult *F. hepatica* cDNA libraries with antisera raised to cysteine proteinase confirmed that the proteinases were Cat L class (60). cDNA cloning encoding secreted cathepsin proteinase has also been isolated. *F. hepatica* was shown to contain multiple genes encoding Cat L, as evidenced by the isolation of multiple PCR fragments encoding Cat L and at least three complete cDNA sequences (61). RT-PCR and degenerative Oligonucleotide primers derived from conserved cysteine proteinase sequence were used to amplify seven different *F. hepatica* cysteine proteinase cDNA clones (62). Five of the clones showed homology to proteinases of the cathepsin L, whereas two appeared to represent the cathepsin B family

In *Fasciola*, the cathepsin L proteinases have been proposed to play a number of functional roles including promoting tissue penetration (56,63,64), nutrient acquisition (65), and egg production (28,66), they are considered to be important targets to which immunoprophylaxis could be directed. In addition, Cat L proteinases purified from the E.S products of *F. hepatica* can prevent the antibody-mediated

attachment of eosinophil to NEJ (67). This indicates that Cat L has an important biological function in immune evasion. Cat L1 and Cat L2 proteinases of adult *F. hepatica* were capable of degrading acid-soluble type III and IV collagen, fibronectin, and laminin (64). The results indicate that the cysteine proteinases secreted by *F. hepatica* may be involved in the process of host tissue invasion

The major secreted proteinases of adult worm are Cat L, while the major proteolytic activity of NEJ is a 29 kD cathepsin B (Cat B) (68). Similar to the Cat L, Cat B can cleave immunoglobulin and therefore may be involed in immune evasion by these invasive stages of liver fluke. It was suggested that Cat B may also play roles in tissue invasion, glycocalyx turnover or excystment of metacercaria (68,69).

Early histochemical studies revealed that proteinase activity was associated with cells of the gut epithelium (70,71). Immunolocalization studies have shown that the Cat L proteinase are synthesized and packaged in vesicles within the caecal epithelial cell of *F. hepatica* (65). In addition, Spithill and Morrison (1997) have demonstrated the presence of Cat L in the caeca and Mehlis' glands of adult *F. hepatica*, and suggest that this enzyme is used for the digestion of nutrient and somehow involved in eggshell formation (66). Cat B was localized to the parenchymal tissue of the adult fluke, but it was localized to the gut lumen and secretory granules within the epithelia in NEJ (68,72).

CHAPTER IV

MATERIALS AND METHODS

1. Collections of Parasite Specimens

1.1 Adult Worms

Adults *Fasciola gigantica* were removed from the bile duct and gall bladders of cattle killed at local abattoirs. They were washed several times in 0.85% NaCl solution to remove blood and bile before being processed further for the experiments.

1.2 Juvenile Worms

Juvenile *F. gigantica* were obtained from Swiss albino mice infected with 150 metacercariae each via stomach tube. The immature parasites were collected from the liver of mice 50 days after infection with *F. gigantica* metacercaria. The juvenile worms were collected and washed several times with 0.85% NaCl solution before using in the experiments.

1.3 Metacercaria

Metacercariae of *F. gigantica* were obtained from *Lymnae ollula* snails infected with *F. gigantica* miracidia in the laboratory. The cercariae were shed from the snails and allowed to settle on the 5 x 5 cm cellophane sheet that were floated on the water surface. After the attachment on cellophane, cercariae encysted themselves

to form metacercarial stage. Metacercariae were observed under stereomicroscope, brushed from the cellophane where the outer cyst of most metacercariae were also removed by brush. The partially excysted metacercariae with the inner transparent cyst wall were washed in 0.85% NaCl solution before being used in the experiments.

2. Primary and Secondary Antibodies Used in Immunolocalization Experiments

2.1 Monoclonal Antibodies

Mouse monoclonal antibody, in the form of culture fluid from hybridoma clones 2D9, which was proven to act against 27 kDa antigen and was tested to be Cat L was provided by Prof. Dr. Vithoon Viyanant, Department of Biology, Faculty of Science, Mahidol university The production protocol was briefly described as follow: Freshly collected worms were washed three times with 0.85% saline to remove blood and bile before incubated in Minimal Essential Medium (MEM) at room temperature for 6-8 hours. Periodically, the MEM medium was collected and replaced whenever its color changed from pink to yellow. The collected solution containing ES antigens was centrifuged and lyophilized. BALB/C mouse was immunized with ES antigen and reimmunized every 2 weeks for about 1 month. After immunization, mouse's spleen cells were removed and fused with P3U1 myeloma cells. Cultures from colonies of hybridoma were screened and rescreened for the desired antibodies by enzyme linked immunosorbant assay (ELISA) and Western blot analysis. The single clone of 2D9 hybridoma was obtained by dilution method. The 2D9 hybridoma clone was expanded and the culture fluid was collected and pooled for furthur analysis 2D9

monoclonal antibody in the culture fluid was confirmed as anti Cat L by it's reaction with 26-27 kDa protein band in immunoblotting analysis (73,74).

2.2. Polyclonal Antibodies

Mouse anti-native *F. gigantica* Cathepsin L and sheep anti-native *F. hepatica* Cathepsin L were obtained from Assoc.Prof. Dr. Terry W. Spithill, Department of Biochemistry and Molecular Biology, Monash University, Clayton, Australia. Briefly, Antisera to the 27-28 kDa complex known to contain Cat L activity were produced by immunizing animals with native Cat L purified from ES antigen by gelatin substrate gel analysis (28). Sera from these animals were collected after 4 weeks of immunization and used as a reagent to identify the presence of Cat L.

2.3 Secondary Antibodies

Fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG antibodies and FITC-conjugated rabbit anti-sheep IgG antibodies were purchased from Sigma Company and Zymed Company respectively.

3. Histological Techniques for Studying the Structure of the Digestive Tract

3.1 Carmine Staining

Metacercariae, 50-day-old juvenile worms and adult *F. gigantica* were washed three times in 0.85% NaCl before the parasites were distended by compression between two glass slides, and immersed in alcohol-formal acetic (AFA) fixative at

room temperature overnight. Then, they were washed in tap water 2 times for 10 minutes each, and dehydrated 1 hour each in 30%, 50% and 70% ethanol. Thereafter, they were immersed in semichon' s carmine for 1 hour, washed in 70 % alcohol, and differentiated in 1% acid alcohol (1% HCl in 70% alcohol), until the pink color was observed. The parasites were then washed in 70% alcohol 2-3 times, and dehydrated for 30 minutes each in 80 %, 95% and 100% ethanol, . Finally, parasites were cleared in xylene, mounted on the glass slides and observed under an Olympus Vanox light microscope.

3.2 Paraffin Technique

Metacercariae, 50-day-old juvenile worms, and adult parasites were fixed in Bouin's solution for overnight. They were washed in 70% ethanol to remove the Bouin's fixative before were dehydrated in a graded series of ethanol (70, 80, 90, 95, 100%) for 30 minutes each, cleared with dioxane, infiltrated and embedded in paraffin wax. Blocks of specimens were sectioned at 5 μm thickness, stained with hematoxylin-eosin before the sections were observed under an Olympus Vanox light microscope.

3.3 Semithin Technique

Metacercariae, 50-day-old juvenile worms and adult parasites were fixed in solution of 4% glutaraldehyde, 2% paraforaldehyde and 0.1 M PBS (pH 7.4) (Kanovsky's fixative) for 2 hours. The fixative was removed by washing with 0.1 M PBS. The specimens were post-fixed in 1% osmium tetroxide in 0.1 M PBS for 1 hour at 4°C, dehydrated in a graded series of ethanol (50-100%) for 15 minutes each. The

specimens were cleared in two changes of propylene oxide (PO), infiltrated in a mixure of propylene oxide and Araldite 502 resin at the ratio of 2:1 for 1 hour and 1:2 for overnight, then embedded in pure Aradite 502 resin for 24 hours. Finally they were polymerized at 45°C and 60°C for 48 hours each, respectively. Blocks of specimens were sectioned at about 1 μm thickness by ultramicrotome Porter Blum MT-2, and stained with methylene blue. The sections were observed and photographed under an Olympus Vanox light microscope.

4. Procedure for Detection of mRNA by *In Situ* Hybridization

4.1 Preparation of the Probe

4.1.1 Isolation of Plasmid DNA from *E. coli* (small-scale preparations of plasmid DNA by the alkaline lysis method)

A transformant *E.coli* XL1-blue clone was provided by Dr. Rudi Grams, Faculty of Allied Health Sciences, Thammasart University and Dr. Suksiri Vichasri Grams, Department of Biology, Faculty of Science, Mahidol University. It carries the pBluescripts SK plasmid (stratagene) into which the coding sequences of a *F. gigantica* Cathepsin-L gene have been subcloned The isolation procedure was performed as follows:

Three ml LB medium were inocculated with a freshly grown transformant *E.coli* colony and incubated overnight at 37°C with vigorous shaking (280 rpm). After centrifugation at 4000 rpm for 5 minutes, the medium was removed, leaving the bacterial pellet as dry as possible The (bacterial) pellet was resuspended in 200 μl of solution I and then transferred to a 1 5 ml tube. After adding 400 μl of

freshly prepared solution II into the sample, mixing carefully by inverting the tube 4 to 5 times, the sample was incubated at 0°C for 5 minutes. Then, 300 µl of chilled solution III was added, mixed carefully and incubated for another 5 minutes at 0°C. The denatured proteins and bacterial DNA were pelleted by centrifugation at 12000 g at RT for 5 minutes. The supernatant containing the plasmid DNA was transferred to a new tube, and 0.6 volumes of isopropanol (2-propanol) were added, mixed and centrifuged at 12000 g at room temperature for 5 minutes. The supernatant was discarded, the pellet was washed with 70% ethanol and centrifuged at 12000 g at room temperature for 5 minutes. The pellet was dried and resuspended in 50 µl of TE buffer and stored at 4°C.

4.1.2 Digestion of Cathepsin L gene from SK-pBluescript Plasmid with Restriction Enzyme

A restriction map was generated for the Cathepsin L gene fragment by using a webcutter 2.0 programe (http://www.firstmarket.com/cutter/cut2.html), and a map and restriction sites of SK-pBluescript was available from the1999 Stratagene catalog (76). Two endonucleases, Hind III and Pst I, were selected for restriction enzyme analysis in the present study. The digesting mixture contained 20 µl of plasmid DNA, 8 µl of 10x React 2Buffer (GibcoBRL), 3 µl Hind III (10 U/µl), 2 µl Pst I (20U/µl), 2 µl RNase and 45 µl water. The resulting DNA fragment were sized separated by electrophoresis in a 0.7% agarose gel cast in Tris-borate-EDTA buffer (77). Restriction fragments of bacteriophage lambda DNA served as size standard.

4.1.3 Isolation of DNA Fragments from Agarose Gel Using QIAquick Gel Extraction Kit

The DNA fragment to be eluted from the gel was determined, followed by cutting the fragment containing piece of gel from the gel with a clean sharp scalpel. The gel slice was weighed in a colorless tube, and then added 300 µl of buffer QG to 1 each 100 mg of gel. Gel was dissolved by incubation at 50°C for 10 minutes, mixed by vortexing the tube every 2-3 minutes during the incubation to help dissolve gel A QIAquick spin column was placed in a provided 2 ml collection tube, the sample loaded to the QIAquick column and centrifuged at 10,000 g for 1 minute. The flow-through was discarded and the QIAquick column was placed back in the same collection tube. Consequently, the DNAs were washed by adding 0.75 ml of PE buffer to QIAquick column and was centrifuged for an additional 1 minute at 10,000 g. The QIAquick column was placed into a clean 1.5 ml microfuge tube. The DNA was eluted by adding 25 µl of 10 mM Tris-HCl, pH 8 5 and distilled water (pH7.5-8.5) to the center of the QIAquick column and centrifuged for 1 min at maximum speed and stored at 4°C.

4.1.4 DNA Labeling Using Random Priming Method

DIG labeled DNA probes were generated enzymatically according to the method of primed labeling (78,79). Following the protocol of the supplier Boehringer, the purified DNA (100 ng) was resuspended in 15 µl of H_2O, then denatured by heating for 10 minutes in a boiling water bath at 100°C and quickly cooled on ice. To the denatured DNA, 2 µl of hexanucleotide mixure, 2 µl dNTP

(dATP, dTTP, dGTP, dCTP and Digoxigenin(DIG)-dUTP) and 1 µl Klenow enzyme were added, mixed well and immediately incubated at 37°C for overnight

4.2 Probe Purification

The labeled DNA was precipitated for 30 minutes at -70°C by adding 2.5 µl 3 M sodium acetate and 75 µl prechilled (-20°C) ethanol. The precipitate was pelleted by centrifuged at 12000 g for 15 minutes. The pellet was washed with 50 µl cold 70% ethanol, dried under vacuum and then dissolved in 50 µl TE buffer. The amount of DIG-labeling was determined by a dot blot assay according to the method in the DIG DNA Labeling and Detection Kit manual.

4.3 Tissue Preparation

Metacercariae, 50-day-old and adult *F. gigantica*, were fixed in 100 mM phosphate buffer containing 4 % paraformaldehyde for 3-4 hours at room temperature. After fixation, the samples were washed in 100 mM phosphate buffer saline (PBS), embedded in paraffin, and cut 5 µm thick. The sections were placed onto gelatin chrome alum-coated glass slides and deparaffinized in fresh xylene twice, for 10 minutes each. The sections were rehydrated twice for 5 minutes each in 100%, 95%, 70% alcohol and DEPC-treated double-distilled water respectively.. The sections were then incubated in DEPC-treated PBS containing 0.1% 20 (PBT) twice, for 5 minutes each and treated with proteinase K (20 µg per ml) in PBT for 20 minutes, at 37°C, followed by DEPC-treated PBT containing 100 mM glycine twice, for 5 minutes each, and finally washed with DEPC-treated PBT twice for 5 minutes

each. Finally, the sections were fixed once again with 4% paraformaldehyde in PBS for 5 minutes and washed in DEPC-treated PBT twice for 5 minutes each.

4.4 Hybridization.

The sections were incubated at 55°C for 10 minutes with prehybridization buffer The buffer was drained from the slides and each section was overlaid with hybridization buffer containing denatured digoxigenin (DIG)-labeled DNA probe (1.40) and covered with a siliconized coverslip. The sections were incubated at 55°C overnight in a humid chamber.

4.5 Posthybridization.

The coverslips were removed from sections by immersing the slides for 10 minutes in 2XSSC, and the sections were washed with 1XSSC, 50% formamide three times, 20 minutes each at 55°C. The sections were finally washed with 1XSSC two times, 15 minutes each at room temperature.

4.6 Immunological Detection

The sections were washed with washing buffer twice for 10 minute each. Then they were incubated with blocking solution for 30 minutes. The blocking solution was decanted and the sections were incubated for 2 hours in a humid chamber with 20 µl alkaline phosphatase-conjugated anti DIG antibody (diluted 1:500 in blocking mixture). The sections were then washed with washing buffer twice, for 10 minutes each, and incubated with detection buffer for 10 minutes. Sections were covered with freshly prepared color substrate solution (detection buffer

containing 20 µl NBT/BCIP mix/ml) and incubated in a humid chamber in the dark, and the color reaction was stop by rinsing the slides with distilled water. The slides were mounted with buffered-glycerol, observed and photographed under an Olympus Vanox light microscope.

5. Indirect Immunofluorescence Method

5.1 Tissue Preparation

Fresh metacercariae, 50-day-old juvenile worms, and adult *F. gigantica* were fixed in 4% paraforaldehyde in 100 mM phosphate buffer saline (PBS) for 4 hours at room temperature. After fixation, the samples were washed in the same PBS solution. Alternatively, another batch of the parasite sample were rapidly frozen by placing it onto a piece of aluminium foil placed over a layer of tissue embedding medium (Tissue Tek OCT, MILES, USA) precooled with liquid nitrogen. Frozen samples were then frozen and stored in liquid nitrogen. Both fixed and frozen samples were sectioned at 5 µm thickness by Leica cryostat Sections were picked up onto a gelatin-chromalum coated microscope slides.

5.2 Immunostaining for Cathepsin L

The sections were further fixed with acetone at -10°C for 10 minutes and air-dried. After washing with 0.01 M PBS for 5 minutes, they were incubated with 0.1% glycine in 0.01 M PBS and 4% BSA in 0.01 M PBS for 15 minutes each to block non-specific binding. The sections were then incubated for 1 hour with primary antibody either the monoclonal antibody clone 2D9 or polyclonal antibodies (mouse anti-native

F. gigantica Cathepsin L and sheep anti-native *F. hepatica* Cathepsin L). The specimens were washed 3 times with PBS for 5 minutes each Subsequently the sections were incubated in secondary antibody (FITC-conjugated goat anti-mouse, FITC-conjugated rabbit anti-sheep) for 30 minutes at room temperature and washed 3 times with PBS for 5 minutes each. The slides were mounted in buffered glycerol, sealed with nail polish, and observed under a Nikon HB 10101 AF fluorescence microscope. For negative control, some sections were processed in the same way, but normal mouse and normal sheep sera were used instead of the antibodies.

CHAPTER V

RESULTS

1. Structure and Histology of the Digestive Tract of *Fasciola gigantica*

1.1 Gross Anatomy

1.1.1 Adult Digestive Tract

The digestive tract of adult *F. gigantica* consists of oral sucker, buccal tube, pharynx, esophagus and highly branched caeca (Figure 2). The oral sucker is located at the anterior tip of the body and is surrounded by the circular, longitudinal and radial muscle fibers. It is connected with the pharynx by the buccal tube, which is a short tubule surrounded by the thin circular and longitudinal muscle fibers. The pharynx is a short muscular tube extending from the buccal tube to the esophagus. The esophagus is surrounded by circular and longitudinal muscle fibers. The caecum is continuous with the esophagus and bifurcated into left and right main caeca extending towards the posterior end of the body. The left and right main caeca give off numerous medial and lateral branches. The proximal part of both caeca, which is anterior to the ventral sucker, has only four lateral branches, but medial branches are not found. The lateral branches of the proximal caecum project towards the anterior cone of body and bifurcate at the end. Posterior to the ventral sucker, both caeca give off medial branches which are short, blind and close to the midline of the body. The medial branches of the left and right main caeca are not connected together, since they

are separated by the main bladder located at the midline of the body. Most of the lateral branches of caeca are long and give off secondary, tertiary, quarternary and more branches projecting towards the lateral and posterior parts of the body (Figure 2)

1.1.2 Fifty-day-old Juveniles' Digestive Tract

The digestive tract of the juvenile stage is similar to that of the adult stage. but the caeca of the adult is more highly branched than that of the juvenile (Figures 4,5). The juveniles' digestive tract consists of oral sucker, buccal tube, pharynx, esophagus and caeca. The oral sucker is connected with pharynx by the buccal tube. The caecum is continuous with the esophagus and bifurcated into two large main caeca. It contains on each side four anterior caecal outgrowths which have bulbous terminations, and some of them show a bifurcation at the end. Posterior to the ventral sucker, the lateral caecal outgrowths have secondary and tertiary branches which extend towards the margin of the body, but the medial outgrowths near the midline are shorter and less developed (Figure 3).

1.1.3 Metacercaria Digestive Tract

The digestive system of metacercariae of *F. gigantica* is fairly well developed. The metacercariae are infective stage of the parasites, which are surrounded by many layers of protective cyst wall. The digestive tract is composed of oral sucker, pharynx, esophagus and lobulated caeca (Figure 4). The oral sucker, which is slightly ventral in position, is connected to a centrally-positioned, oval, muscular pharynx that has a thick wall and a slit-like lumen. The esophagus is bifurcated into large lateral caeca situated middorsally. The lobulated caeca curve

round the ventral sucker, which is situated in the posterior half of the body. The two caeca are located ventrally and near the midline, and they end blindly close to the posterior tip of the parasites' body.

1.2 Histology of the Digestive Tract

1.2.1 The Epithelium of Digestive Tract of 50-day-old Juvenile and Adult Stages

The epithelium of digestive tracts of 50-day-old *F. gigantica* is similar to that of the adult stage It consists of 2 types of epithelia: the tegumental-type which is a syncytium similar to the tegument, and the digestive-type which is composed of an array of cuboid digestive cells which are mostly found in the caecum The epithelium plays a role in nutrient digestion and absorption. The proximal part of digestive tract, including oral sucker, buccal tube, pharynx, esophagus and part of the tract anterior to the caecal bifurcation are lined by tegumental-type epithelium (Figure7A,B, 8A,B), whereas the caecum that is the major part of the digestive tract is lined by digestive-type epithelium (Figure 8D, 10A-D). At the caecal bifurcation, there is the transition point from tegumental type to digestive type epithelium, and both types show markedly different characteristic (Figure 7C,D, 8C, 9C,D).

The tegumental-type epithelium is characterized by numerous basal infolding extending towards the apical surface which could be identified by the corrugation of cross section of ridges which appear like microvilli (Figure 9A-D).

The digestive-type epithelium is composed of columnar or cuboidal cells with round and basally located euchromatic nuclei. The cells have prominent nucleoli and their apical surface have numerous stereocilia (Figure 11A-D, 12A-D).

Moreover, the digestive-type epithelial cells can be classified into 3 types according to their staining properties described as follows:

Type-1 epithelial cells: In the 50-day-old juvenile stage, the cells are generally cuboidal in shape and show many stereocilia (Figure 12B). However, some cells possess long apical processes with tufts of stereocilia. The cytoplasm is densely stained and contain numerous dense granules. In adult stage, the type-1 epithelial cells are tall columnar in shape and have oval nuclei with many blocks of heterochromatin (Figure 11C). However, in the distended caecum the cells are flattened and have cuboidal shape (Figure 13A).

Type-2 epithelial cells: In juvenile stage, the cells are cuboidal in shape and have many apical stereocilia. The cytoplasm is moderately stained and appear paler than that of the type 1 epithelial cells. The number of dense granules is lower than in type-1 epithelial cells. (Figure 12B,C). In adult stage, the type-2 epithelial cells are moderately stained, and the cell are columnar in shape and have oval nuclei (Figure 11B).

Type-3 epithelial cells: In the juvenile stage, the cells are cuboidal in shape and have some stereocilia (Figure 12D). The euchromatic nuclei are round and contain prominent nucleoli. The cytoplasm is vacuolated and shows dense granules scattered throughout the cell In the adult stage, the type-3 epithelial cells are columnar in shape and possess closely-packed stereocilia (Figure 11C). In some areas, the cells show ditended apical cytoplasm which appear vacuolated, while the basal cytoplasm is lightly stained (Figure 11D)

All three types of epithelial cells are found in the same area, but the number of each cell-type depends on the amount of food in the caecal lumen. If the

caecal lumen contains a lot of food, type 3 epithelial cell is found more frequently and the caecal lumen is dilated, while the other cell types are fewer in number (Figure 11A). When the food in the caecal lumen is scanty, type-3 epithelial cells also decrease in number, while the type-1 caecal epithelial cells are more numerous (Figure 13B,C). The type-1 epithelial cell is mostly found when caecal lumen is empty, and the caecum appears contracted. The epithelium is relaxed so that their apical brances become so tightly interdigitated that the lumen is obliterated (Figure 13D).

1.2.2 Metacercarial Stage

The epithelia of oral sucker, pharynx and esophagus in metacercariae are of tegumental type (Figure 14A). The caecal epithelial cells of metacariae are quite different from those of the adult and juvenile. These cells are completely filled with many large oval and very dense granules in comparison to those of adult and 50-day-old juvenile. Each caecal lumen appears very irregular in shape, and is surrounded by only a few epithelial cells. The euchromatic nuclei are located in basal part of the cells and show a prominent nucleolus (Figure 14B).

2. Expression of Cathepsin L Gene in the Digestive Tract of *Fasciola gigantica* as Studied by *In Situ* Hybridization

2.1 Expression of Cat L in Adult *Fasciola gigantica*

In adult *F. gigantica*, Cat L mRNA was predominantly localized in the cytoplasm of the caecal epithelial cells, but was not detected in their nuclei (Figure 15B-D). The positive reaction was observed in form of small fine granules in the cytoplasm, and was found in all 3 types of caecal epithelial cells. Negative control sections, using hybridization buffer, did not show any reaction (Figure15A). At caecal bifurcation, the transition point from tegumental type to digestive type epithelium begins and Cat L mRNA was present only in the digestive-type epithelium, whereas the tegumental-type epithelium did not show any positive staining (Figure 16C-E).

The tegumental-type epithelium covering the remaining parts of the digestive tract including oral sucker, buccal tube, pharynx and esophagus did not exhibit the presence of Cat L mRNA (Figure 16A-B). None of the tissue of reproductive system including testis and uterus were stained (Figure 17A-D).

2.2 Expression of Cat L in Juvenile *Fasciola gigantica*

In 50-day-old juvenile of *F. gigantica*, Cat L mRNA was localized in the cytoplasm of the caecal epithelium (Fig18B-D), while the control sections showed no staining (Figure 18A). At the transition point from tegumental-type to digestive-type epithelium of the caecal bifurcation, Cat L mRNA was detected only in the digestive-type epithelium similar to the adult stage (Figure 19C-D). The other parts of the

digestive tract, including pharynx, ventral sucker and Mehlis' gland, showed no positive reaction (Figure 19A-B, 20A-D).

2.3 Expression of Cat L in Metacercarial Stage of *Fasciola gigantica*

Unlike in juvenile and adult stage Cat L mRNA could not be detected in the digestive epithelium and other tissues of metacercaria (Figure 21).

3. Localization of Cathepsin L in the Digestive Tract of *Fasciola gigantica*

3.1 Localization by Using Monoclonal Antibody

MoAb 2D9, a monoclonal antibody to Cat L, at molecular weight 26-27 kDa was used as primary antibody to react with the specific antigen. It was found that for the adult worm strong fluorescence was observed in forms of large intensely stained globules and very fine granules both in the central part of the caecal lumen and in the apical cytoplasm of caecal epithelial cells (Figure 22B,C). In addition, stereocilia were intensely stained, while muscle cells around the caecum, parenchyma and other tissues were not stained (Figure 22D). Control experiment using culture fluid or other monoclonal antibodies did not give positive fluoresence (Figure 22A). However, protein globules in the vitelline cells showed yellowish autofluorescence.

3.2 Localization by Using Polyclonal Antibodies

Mouse anti-native *F. gigantica* cathepsin L and sheep anti-native *F. hepatica* cathepsin L polyclonal antibodies were used as primary antibody probe in frozen

sections of *F. gigantica* tissues. It was found that positive reaction was observed with both antibodies..

3.2.1 Localization of Cat L in Adult Worm Tissues

In adult worm section, the enzyme Cat L was localized only in caecal epithelial cells. The cytoplasm of epithelial cells showed bright fluorescence that was finely granular (Figure 24A-D, 25A-D). Some sections show abundant granules in the apical region of the epithelial cells and lumen. The epithelium covering other parts of the digestive tract including oral sucker, buccal tube, pharynx, esophagus which are tegumental-type epithelium, shows weakly non-specific fluorescence staining (Figure 26A, 27A). At the transition point from tegumental type to digestive type epithelium at the caecal bifurcation, strong fluorescence was observed only in the digestive-type epithelium, especially in the cytoplasm, whereas the tegumental-type epithelium showed non-specific fluorescence staining at the outer surface of the epithelium (Figure 26C-D, 27A-D). The tegument shows non-specific staining fluorescence, especially at the outer surface and spines. No immunoreactivity for enzyme Cat L was seen in nuclei of the epithelial cells, and the muscles surrounding the caeca and parenchyma. Control sections treated with normal mouse and normal sheep sera showed no fluorescence staining over any part of other organs, but non-specific staining at the apical surface of the tegument and spine were seen (Figure 23A-D).

3.2.2 Localization of Cat L in 50-day-old Juveniles' Worm Tissues

Sections of the caeca of 50-day-old juvenile *F. gigantica* were found to give strong flurescence with both antibodies. The positive reaction was localized

mainly in the cytoplasm of the caecal epithelial cells and appeared in the form of very fine granules, while the nuclei were not stained (Figure 28B-D, 29B-D). Some sections show accumulation granules at the uppermost part of the apical cytoplasm and lumen. The tegument showed weak non-specific staining on the outer surface. Control sections treated with normal mouse and normal sheep sera showed no fluorescence staining in most parts of the organ system excepted that weak non-specific staining was observed at the apical surface of the tegument and spine (Figure 28A, 29A).

3.2.3 Staining of Metacercarial Tissues

Unlike juvenile and adult stages, metacercariae of *F. gigantica* did not exhibited specific fluorescence staining in any tissues by both antibodies. However, non-specific fluorescence was observed on the cyst wall surrounding the metacercaria when compared to the control sections (Figure 30A,B).

Figure 2. Whole mount of adult *F. gigantica* stained by carmine showing oral sucker (Os), pharynx (Ph), caecal bifurcation (Cb), caecum (Ca), ventral sucker (Vs), uterus (Ut), ovary (Ov), Mehlis' gland (Mg), testis (Ti) and bladder (Bl).

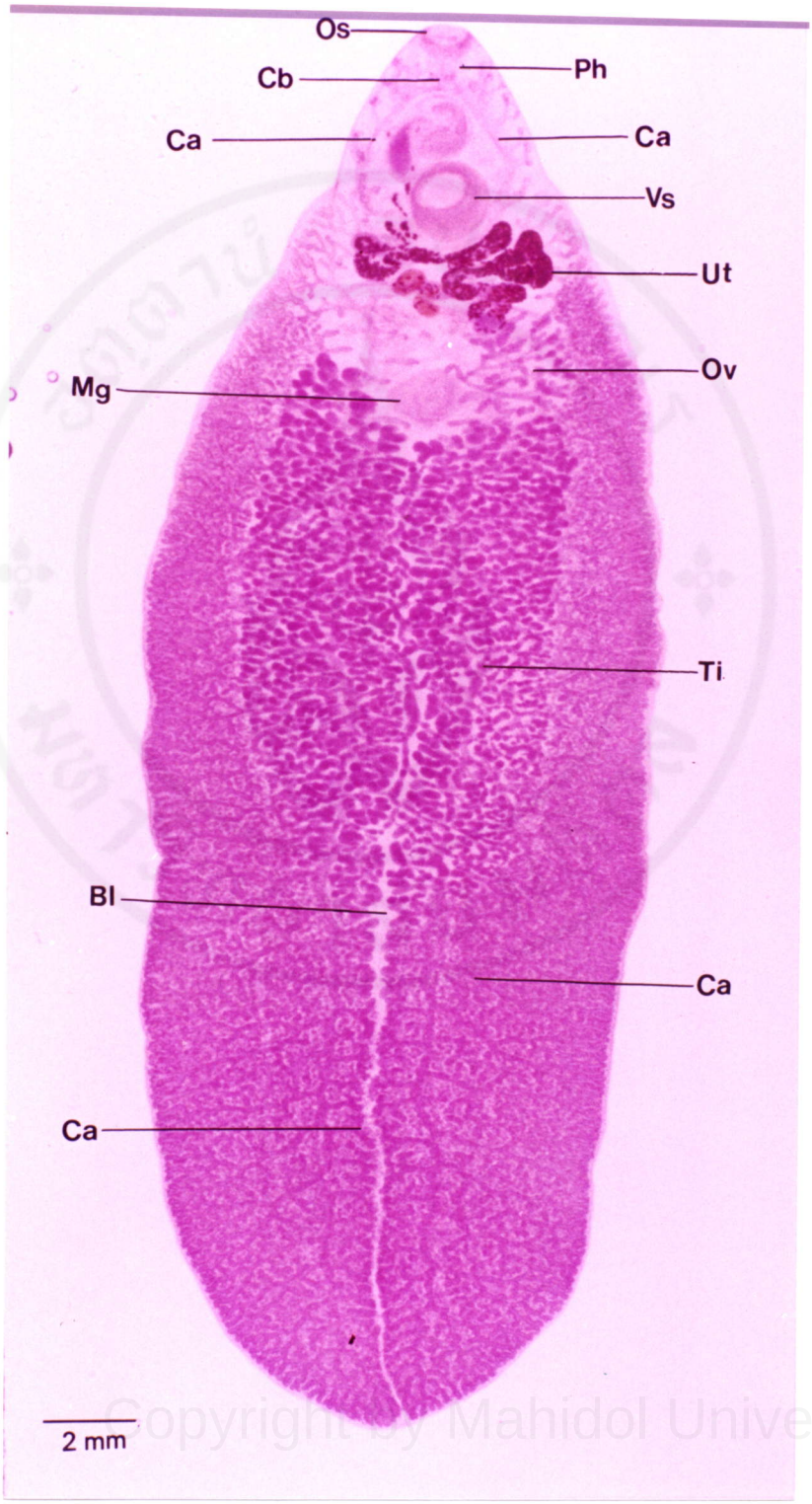


Figure 3. Whole mount of 50-day-old juvenile *F. gigantica* stained by carmine showing digestive organs: oral sucker (Os), pharynx (Ph), caecal bifurcation (Cb) and caecum (Ca). The reproductive system is composed of primodial genital organ: uterus (Ut), seminal vesicle (Sv), Mehlis'gland (Mg), testis (Ti) and prostate gland (Pg). Vs = ventral sucker, Bl = bladder.

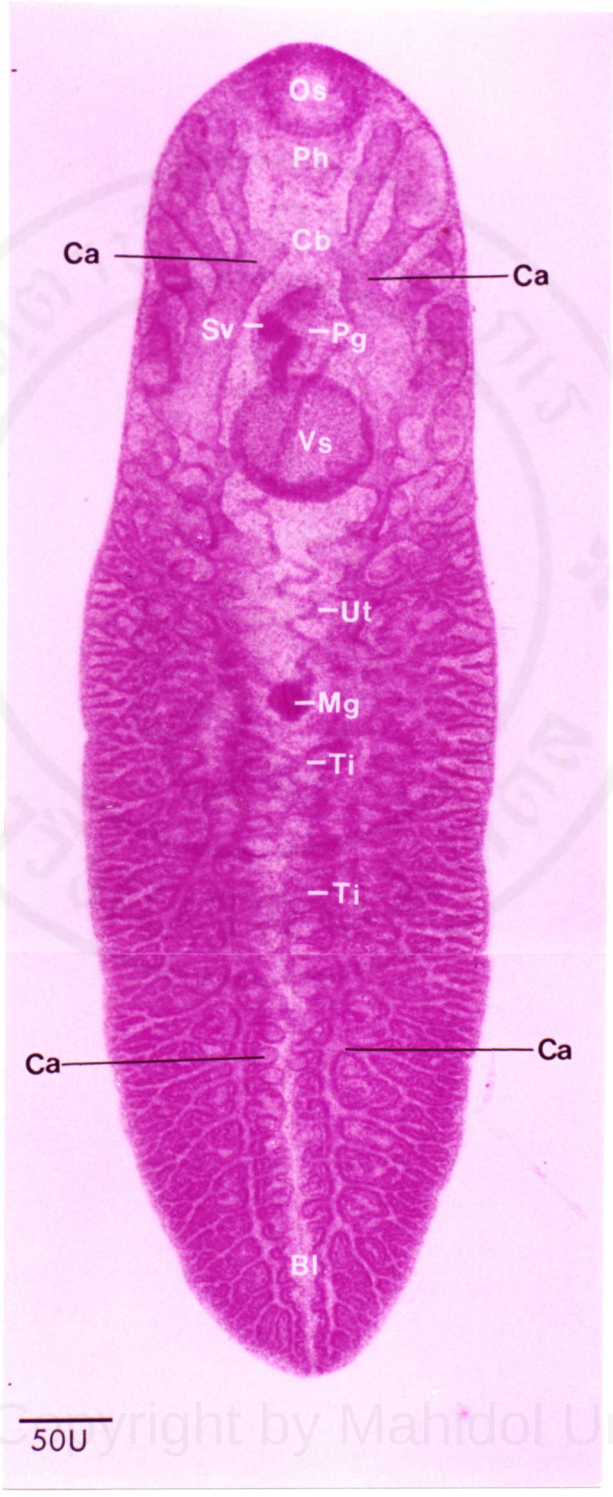


Figure 4. Photomicrograph of living metacercaria and newly excysted juvenile (NEJ).

A) Whole mount of the encysted metacercaria (upper) showing the oral sucker (Os), pharynx (Ph), caecum (Ca), ventral sucker (Vs), bladder (Bl) and protective cyst wall (Cw2). The lower structure is the remaining cyst after the excystment of the metacercaria.

B) Whole mount of newly excysted juvenile (NEJ) *F. gigantica* showing the oral sucker (Os), pharynx (Ph), lobulated caecum (Ca), ventral sucker (Vs) and bladder (Bl).

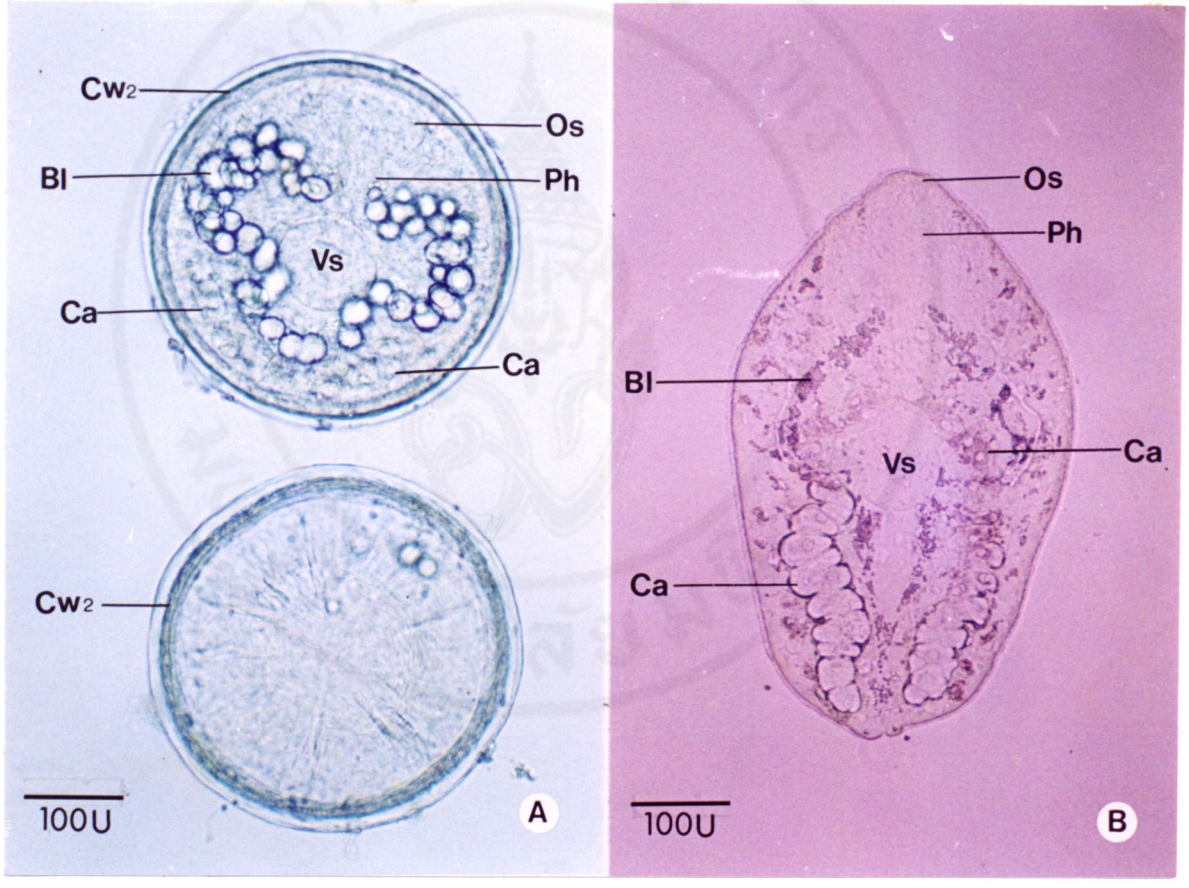


Figure 5. Paraffin sections of the adult stained with H&E.

A) Parasagittal section showing oral sucker (Os), ventral sucker (Vs) seminal vesicle (Sv), uterus (Ut), ovary (Ov), testis (Ti), Mehlis' gland (Mg), caecum (Ca), bladder (Bl) and vitelline gland (Vi).

B) Cross section at level B in Fig. A showing the seminal vesicle (Sv) and ventral sucker (Vs)

C) Cross section at level C showing the vitelline gland (Vi), uterus (Ut) and ovary (Ov).

D) Cross section at level D showing the Mehlis' gland (Mg), caecum (Ca), bladder (Bl), vitelline gland (Vi) and testis (Ti).

E) Cross section at level E showing the caecum (Ca), testis (Ti) and vitelline gland (Vi).

F) Cross section at level F showing the caecum (Ca) and testis (Ti).

G) Cross section at level G showing the bladder (Bl) and vetilline gland (Vi).

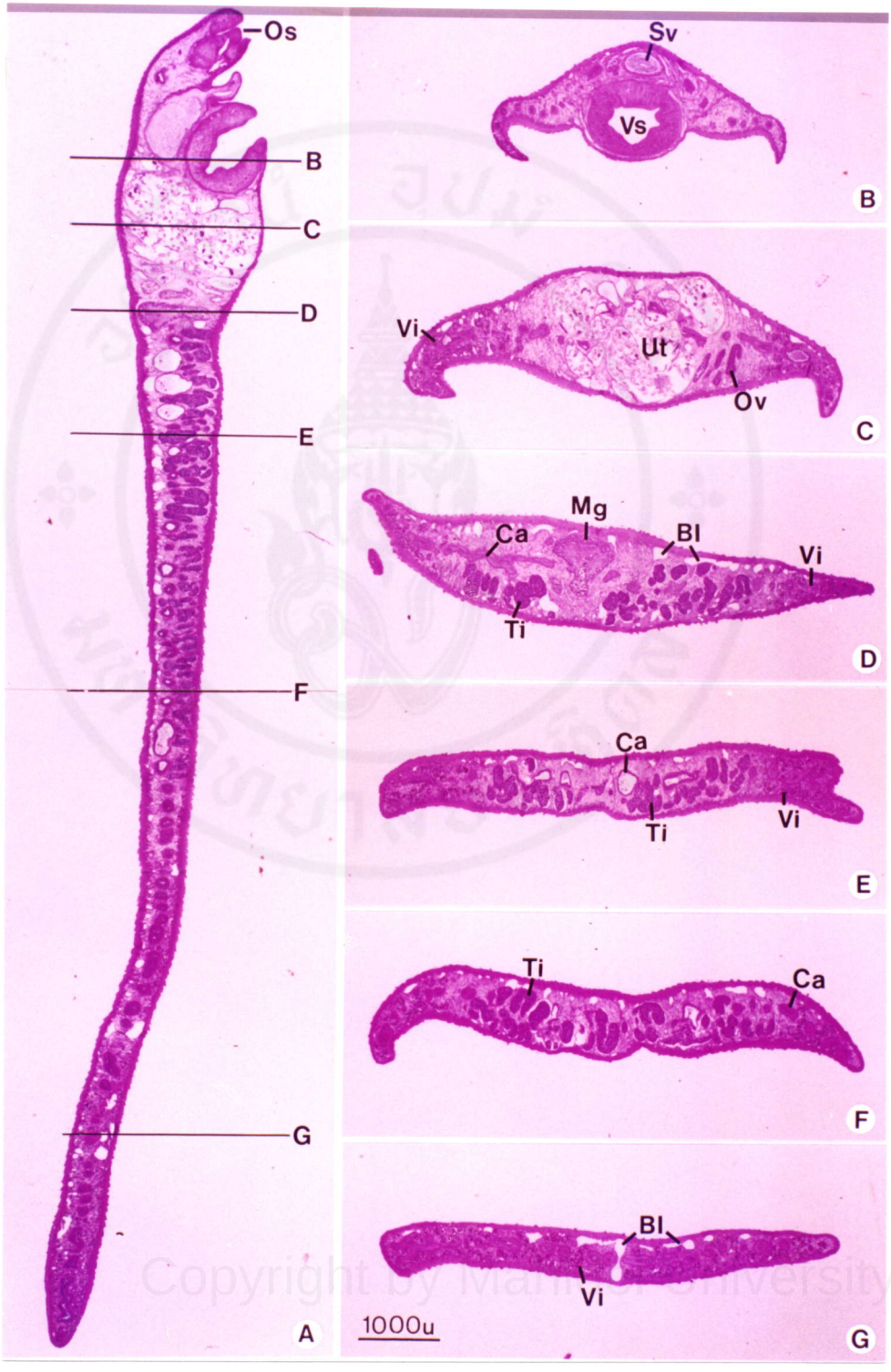


Figure 6. Paraffin sections of a 50-day-old *F. gigantica* stained with H&E.

A) Cross section of the oral sucker (Os) showing the pharynx (Ph) and caecum (Ca)

B) Cross section of the anterior part showing the caecal bifurcation (Cb) and caecum (Ca)

C) Cross section of the ventral sucker (Vs) showing the caecum (Ca) and primodial of seminal vesicle (Sv), prostate gland (Pg).

D) Cross section of the middle part showing the caecum (Ca) and primodial of Mehlis' gland (Mg), ovary (Ov).

E&F) Cross section of the posterior part showing the caecum (Ca) and primodial of testis (Ti).

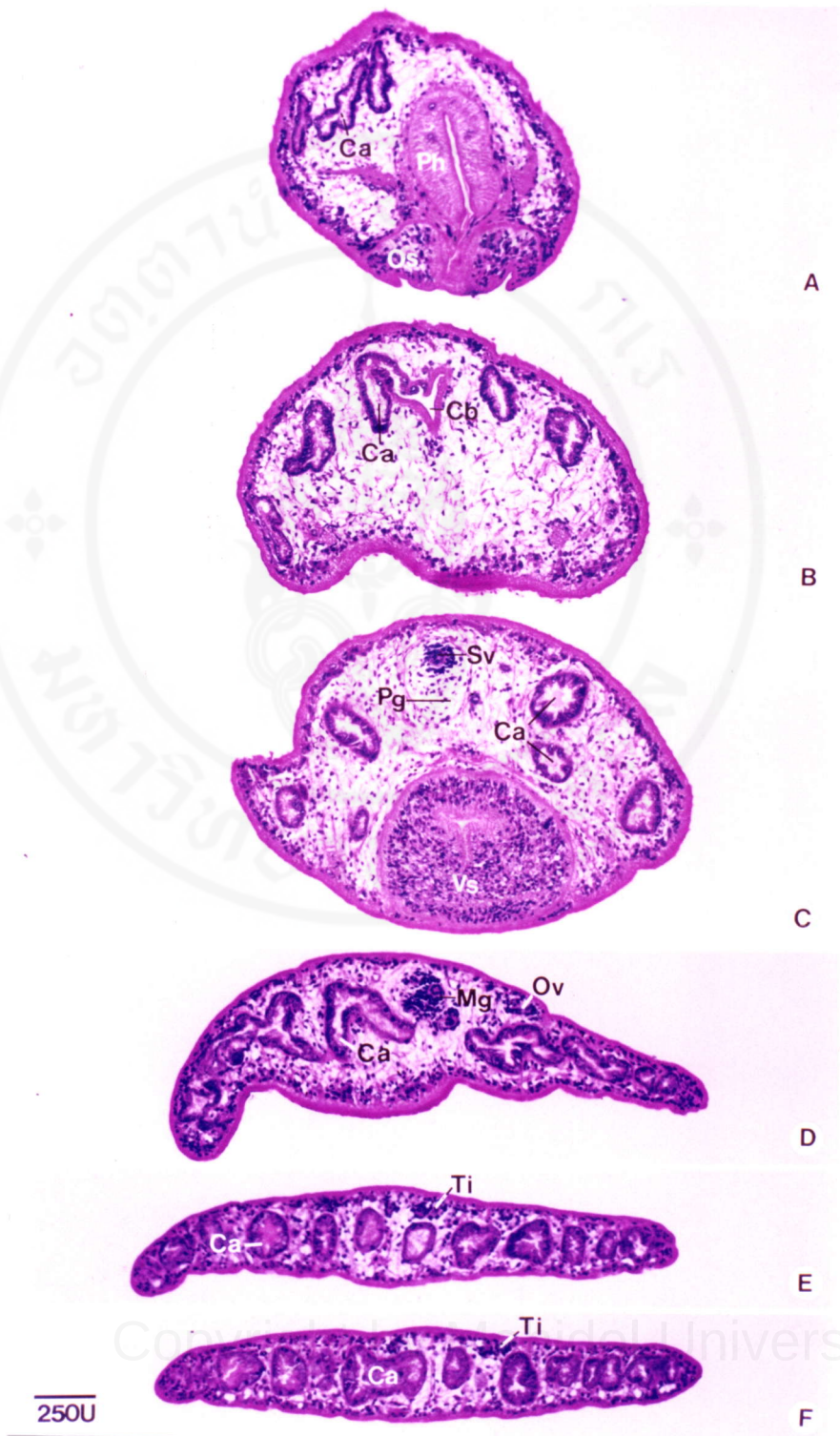


Figure 7. Paraffin sections of the proximal part of the digestive tract of adult *F. gigantica* stained with H&E.

A) Cross section of the oral sucker (Os) showing tegumental-type epithelium (Ep).

B) Cross section of the pharynx (Ph) showing the tegumental-type epithelium (Ep).

C) A low-power micrograph of the caecal bifurcation (Cb) showing the transition point (arrows) from the tegumental-type to digestive-type epithelium.

D) Higher magnification of the transition point (arrows) showing columnar cells of the digestive-type epithelium (Ca).

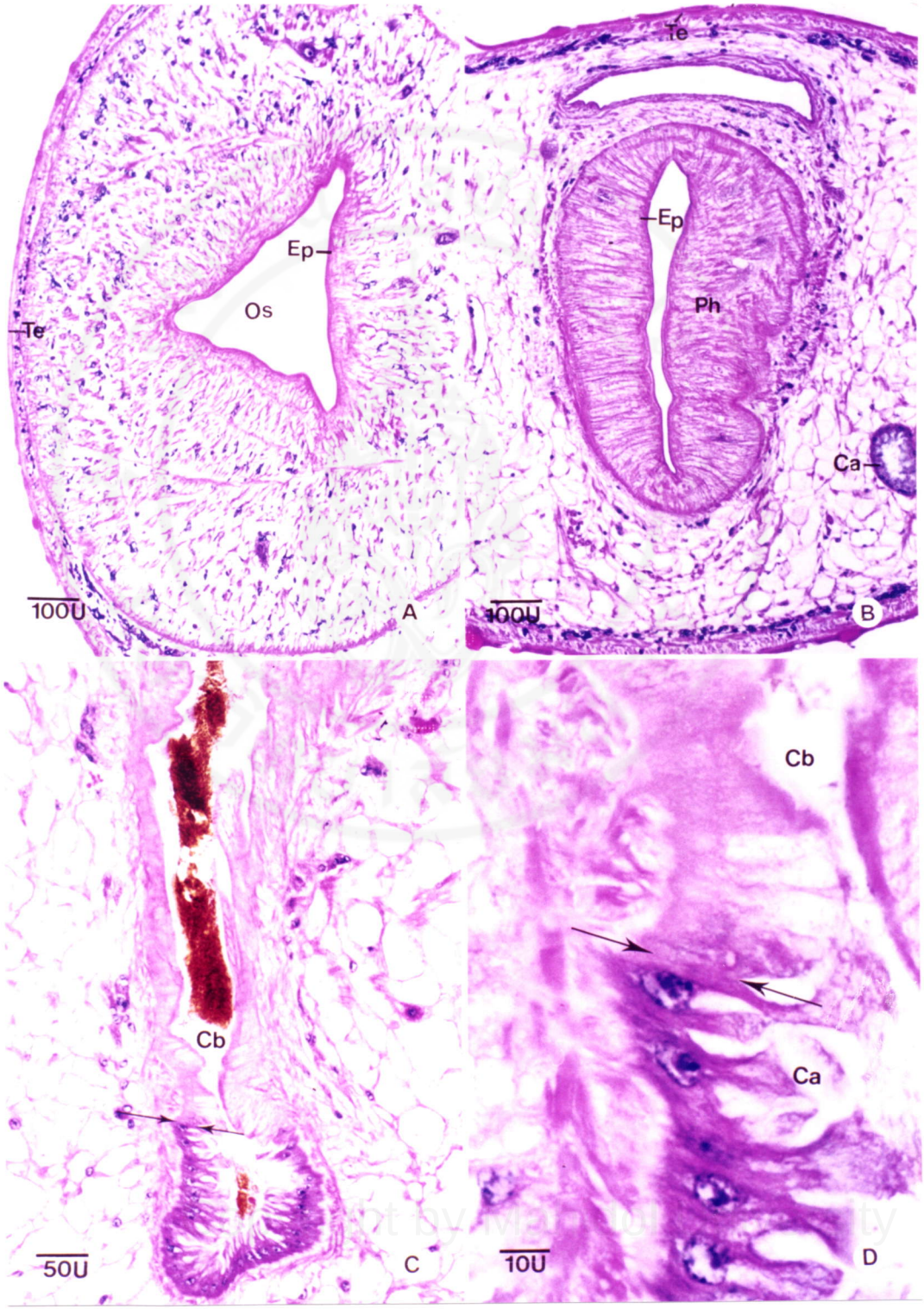

Te
Ep
Os
100U
A
Te
Ep
Ph
Ca
100U
B
Cb
50U
C
Cb
Ca
10U
D

Figure 8. Paraffin sections of the digestive tract of 50-day-old *F. gigantica* stained with H&E.

A) Cross section of oral sucker (Os) showing tegumental-type epithelium (Ep).

B) .Cross section of pharynx (Ph) showing tegumental-type epithelium (Ep).

C) A high-power micrograph of the part distal to the esophagus showing caecal bifurcation (Cb), and the transition point (arrows) of alternation from tegumental-type to digestive-type epithelium.

D) Cross section of the middle part of the parasite showing caeca (Ca) whose lumina are lined by cuboidal digestive-type cells.

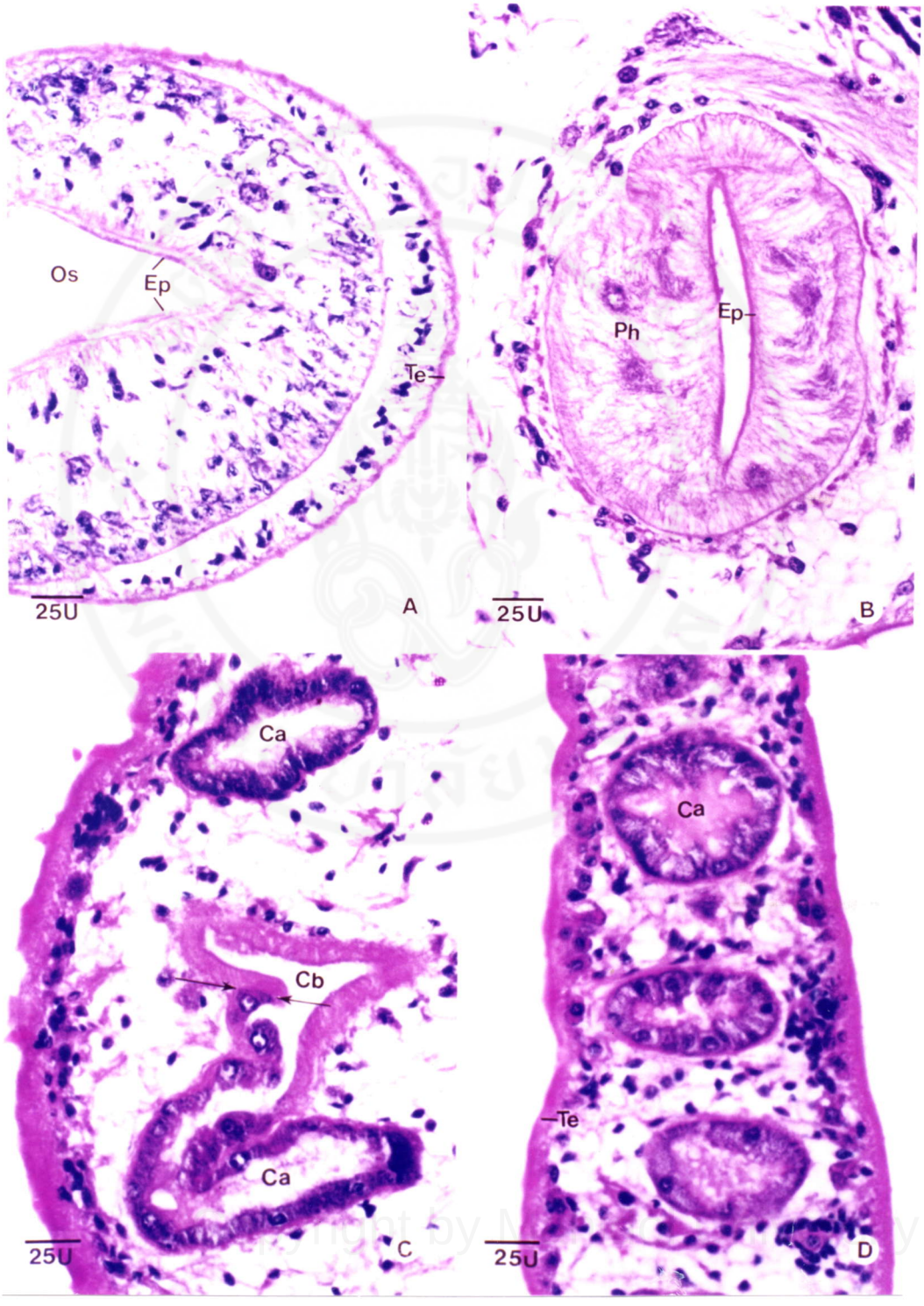


Figure 9. Semithin sections of the proximal part of the digestive tract of 50 day-old *F. gigantica* stained with methylene blue.

A) A low-power micrograph of a cross section of the anterior cone of the parasite showing pharynx (Ph) and caeca (Ca).

B) Higher magnification of the pharynx in Fig. A showing tegumental-type epithelium (Ep).

C) An enlargement of the bifurcation (Cb) of the caecum in Fig. A showing the transition point (arrows) of epithelium alternation.

D) Higher magnification of the transition point (arrows) in Fig. C showing two types of epithelia. The tegumental-type epithelium (Ep) is characterized by numerous basal infolding which is corrugated with cross section of ridges which appear like microvilli. Digestive-type epithelium (Ca) is cuboidal in shape and has many stereocilia.

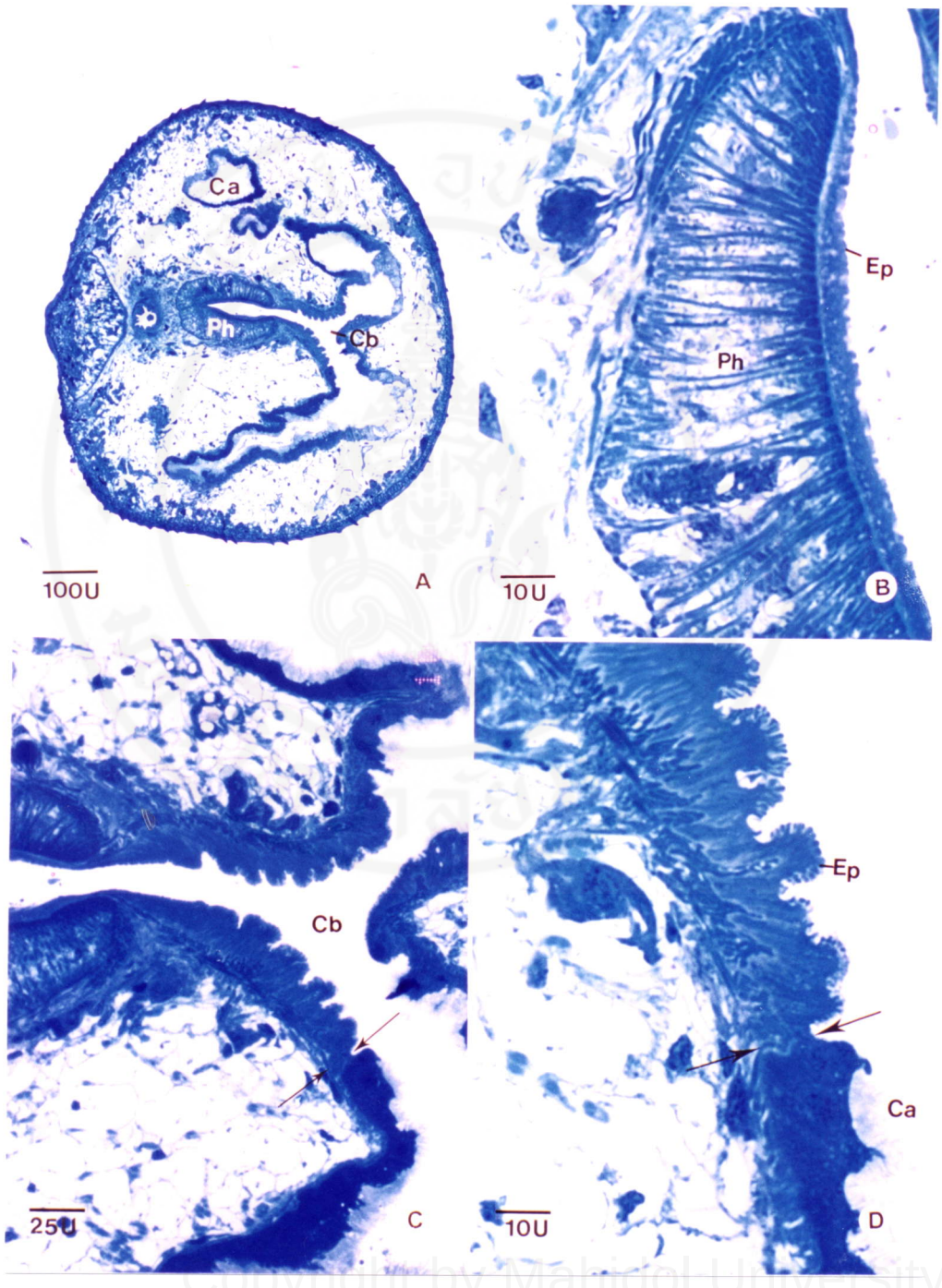


Figure 10. Paraffin sections of the caecum of adult *F. gigantica* stained with H&E.

A) A low-power micrograph of a cross section of a caecum (Ca) showing cuboidal epithelium that has numerous stereocilia.

B) A high-power micrograph of the caecum (Ca) in Fig. A showing type-1 epithelial cell (Ep1). The cell is cuboidal in shape and its apical surface has numerous stereocilia. The cytoplasm is densely stained.

C) A high-power micrograph of caecum (Ca) in Fig. A showing type-2 epithelial cell (Ep2). The cell has cuboidal in shape but the cytoplasm is moderately stained.

D) A high-power micrograph of another caecum (Ca) showing type-3 epithelial cell (Ep3) which is cuboidal in shape and the cytoplasm is lightly stained.

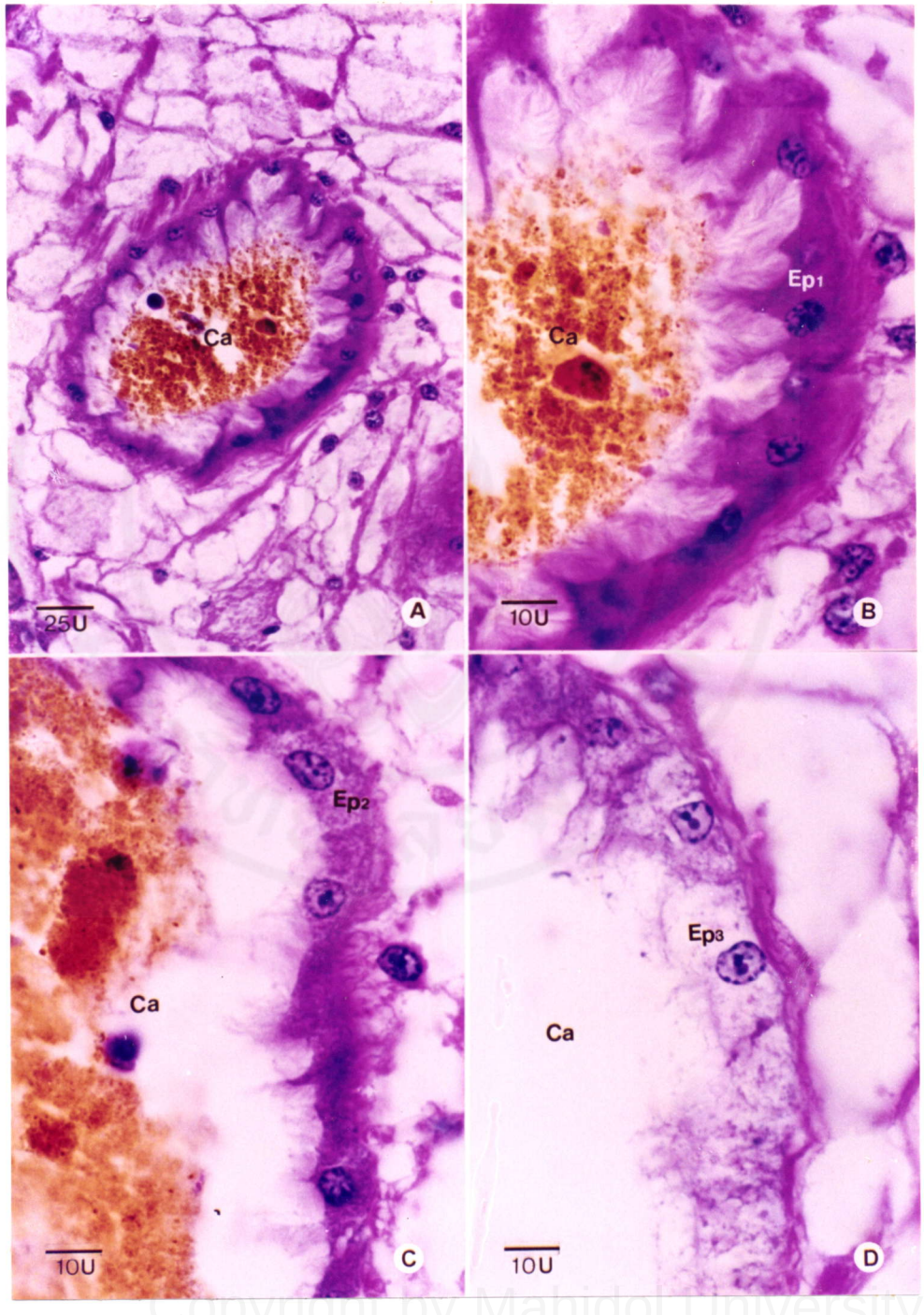


Figure 11. Semithin sections of cross section of the posterior part of adult *F. gigantica* stained with methylene blue.

A) A lower-power micrograph of the posterior part of the body of *F. gigantica* showing a cross section of caecum (Ca) which is lined by columnar epithelium. Vitelline gland (Vi) and bladder (Bl) are also shown.

B) A high-power micrograph of a part the caecum (Ca) showing type-1 epithelial cell (Ep1) The cell is tall columnar in shape and has oval nucleus with many block of heterochromatin. The cytoplasm is densely stained. Type-2 epithelial cell (Ep2) has similar shape, but the cytoplasm is moderately stained.

C) A high-power micrograph of caecum (Ca) showing type-3 epithelial cell (Ep3) which is columnar in shape and possess closely-packed stereocilia (arrows). The cytoplasm is lightly stained.

D) A high-power micrograph of caecum (Ca) in Fig. A showing type-3 epithelial cell (Ep3). The cell has distended apical cytoplasm which appears vacuolated, and the basal cytoplasm shows longitudinal striations.

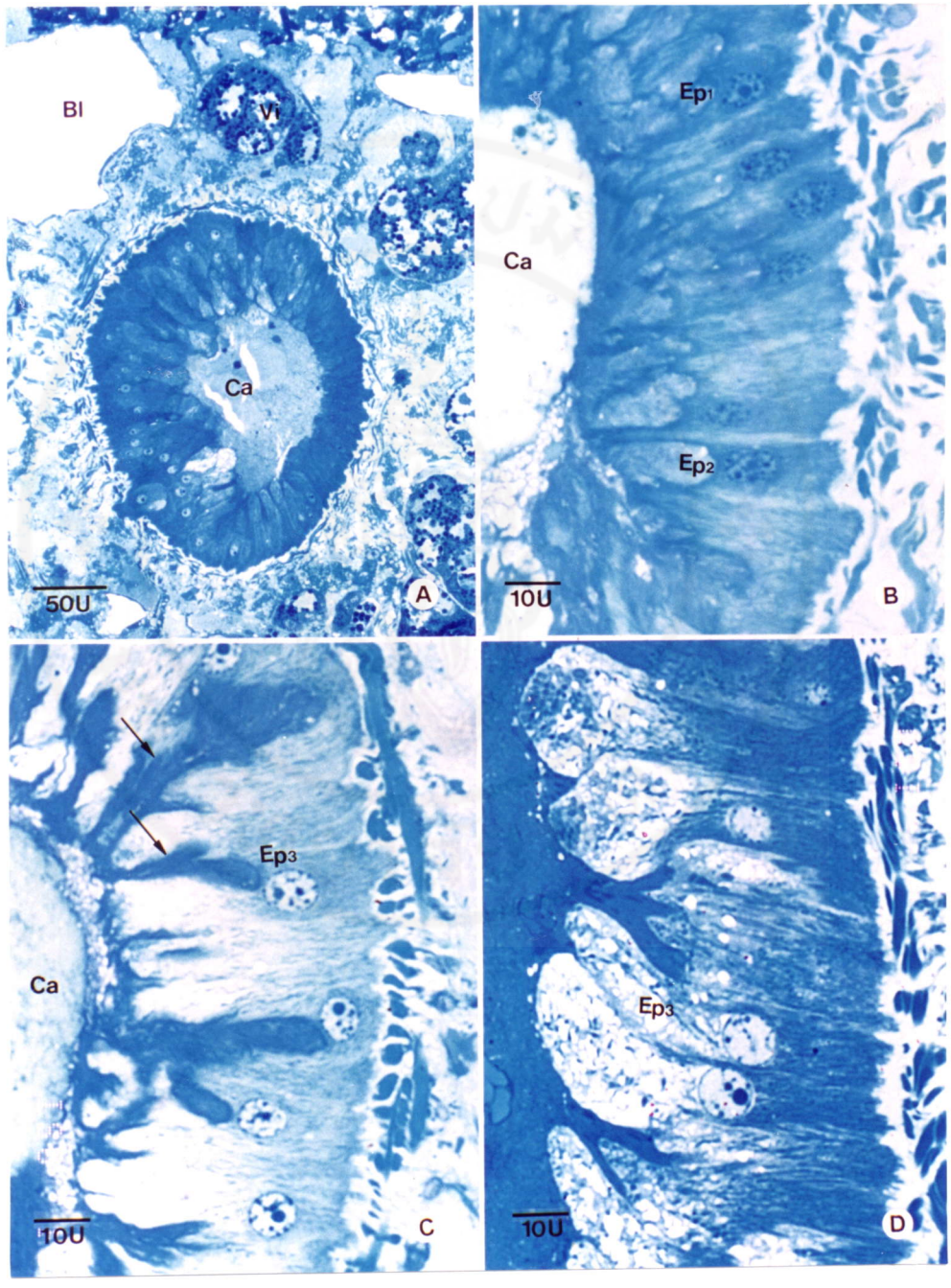


Figure 12. Semithin sections of cross sections of the caecum of 50-day-old *F. gigantica* stained with methylene blue.

A) A low-power micrograph of caecum (Ca) showing that the epithelium is composed of cuboidal cells with round euchromatic nuclei.

B) A high-power micrograph of caecum (Ca) showing type-1 epithelial cell (Ep1) the cell which is cuboidal in shape and shows many long stereocilia. The cytoplasm is densely stained and contained numerous dense granule. Type-2 epithelial cell (Ep2) is also cuboidal in shape, but has larger size. The cytoplasm is moderately stained. Type-3 epithelial cell (Ep3) has siminar size and shape to type-2 cell, but the cytoplasm is lightly stained and shows dense granule.

C) A high-power micrograph of caecum (Ca) in Fig. A showing type-2 (Ep2) and type-3 epithelial cells (Ep3) which are quite different in size and staining intensity.

D) A high-power micrograph of another part of the caecum in Fig. A showing type-3 epithelial cell (Ep3). The cell is low cuboidal in shape and has fewer stereocilia than those of the type-2 cell. The euchromatic nuclei is round and contains a prominent nucleolus. The cytoplasm is vacuolated and shows dense granules scattered through out the cell (arrows).

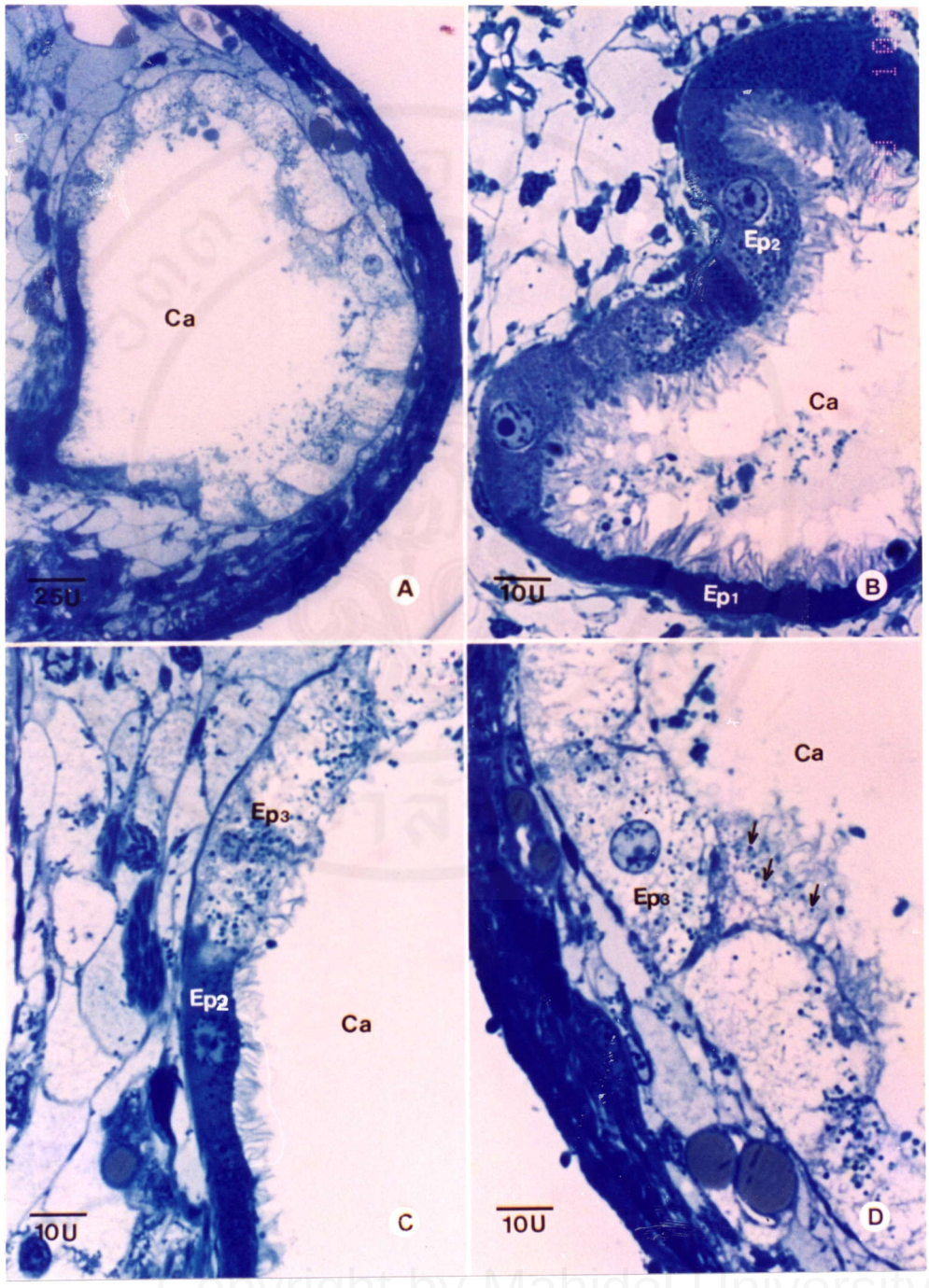


Figure 13. Semithin sections of cross sections of the caecum of adult *F. gigantica* stained with methylene blue.

A) A low-power micrograph of the caecum with a lot of food in the lumen, showing that type-3 (Ep3) epithelial cells are more numerous than type-1(Ep1) and type-2 (Ep2) cells.

B,C) High-power micrographs of another cross section of caecum with some food present in the lumen, showing that the number of type 3 (Ep3) epithelial cells is decreased, while the number of the type-1 epithelial cells (Ep1) is increased.

D) A high-power micrograph of another cross section of caecum with empty lumen showing that most cells in the epithelium are of type-1 cells (Ep1).

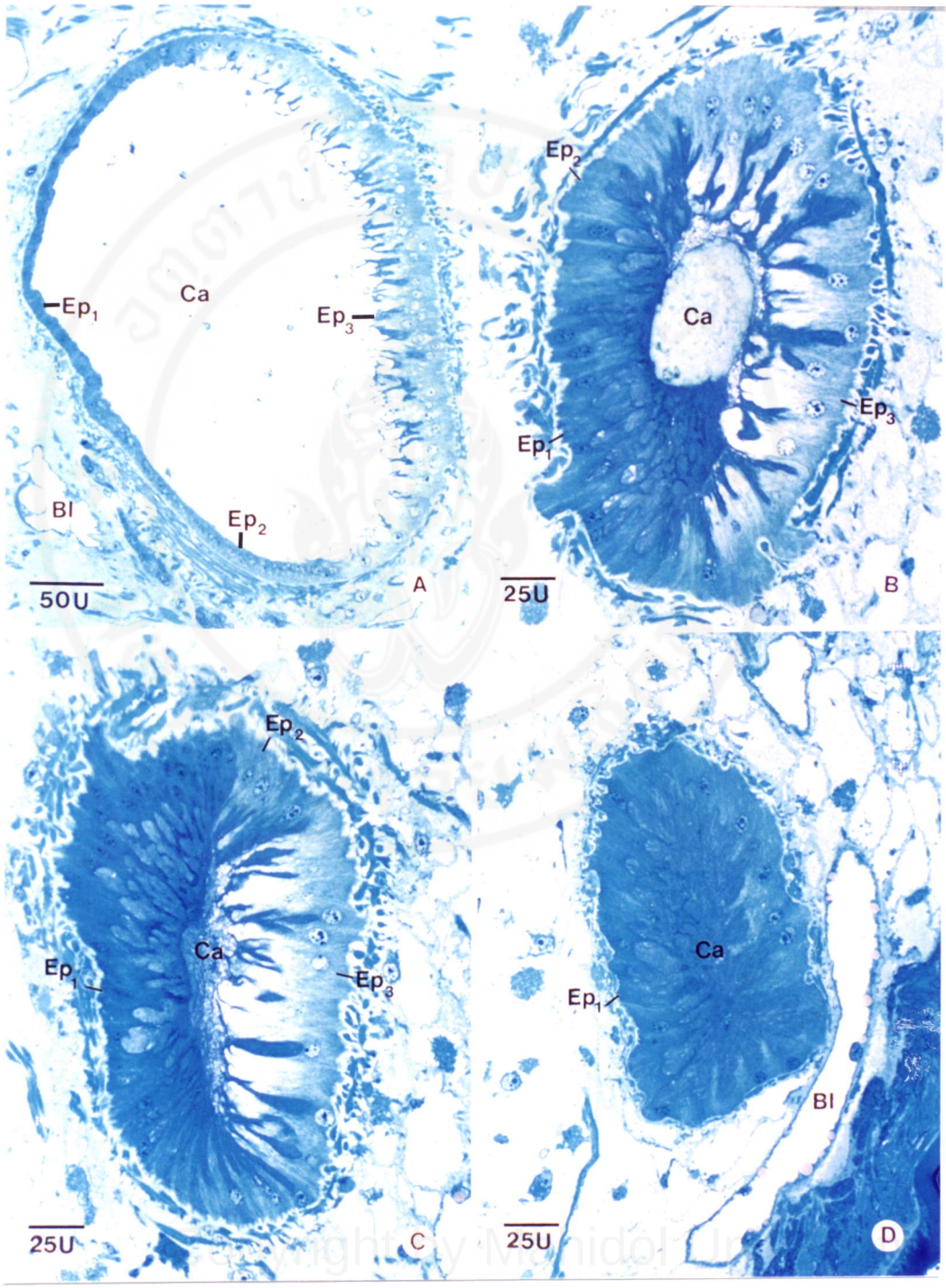


Figure 14. Semithin sections of the metacercariae of *F. gigantica* stained with methylene blue

A) A high-power micrograph of the anterior part of the metacercariae showing the oral sucker (Os) and pharynx (Ph) both of which possess tegumental-type epitheluim. Vs = ventral sucker, Bl = bladder, Sp = spine, Te = tegument

B) A high-power micrograph of the middle part of the metacercariae caecum (Ca) showing that the caecal epithelial cells are filled with large and very dense granules (arrows). The caecal lumen appears very irregular in shape, and is surrounded by only a few epithelial cells. Bl = bladder, nu = nucleus

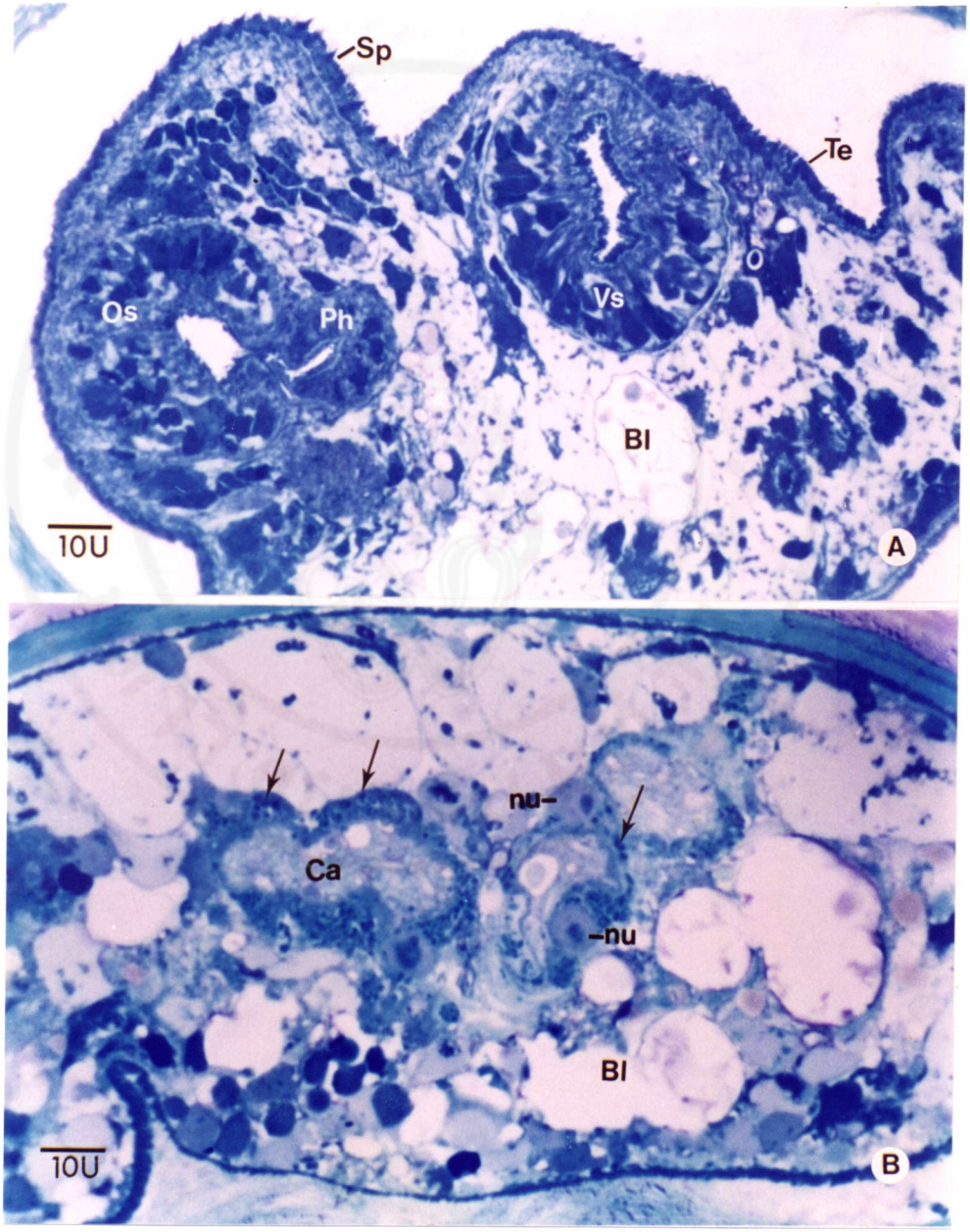


Figure 15. Localization of Cat L mRNA in paraffin sections of the posterior part of adult *F. gigantica* by *in situ* hybridization using DIG-labeled cDNA probe.

A) Control section stained with hybridization buffer instead of probe showing no positive staining in the caecum (Ca), vitelline gland (Vi) and tegument (Te).

B) Cross section stained with the probe showing positive staining specifically in the caecum (Ca), while the vitelline gland (Vi) and tegument (Te) are negatively stained.

C) A high-power micrograph showing positive staining in the cytoplasm of the caecal epithelial cell (Ca).

D) Higher magnification showing positive staining in the cytoplasm of the caecal epithelial cell (Ca), while the nuclei (nu) are not stained.

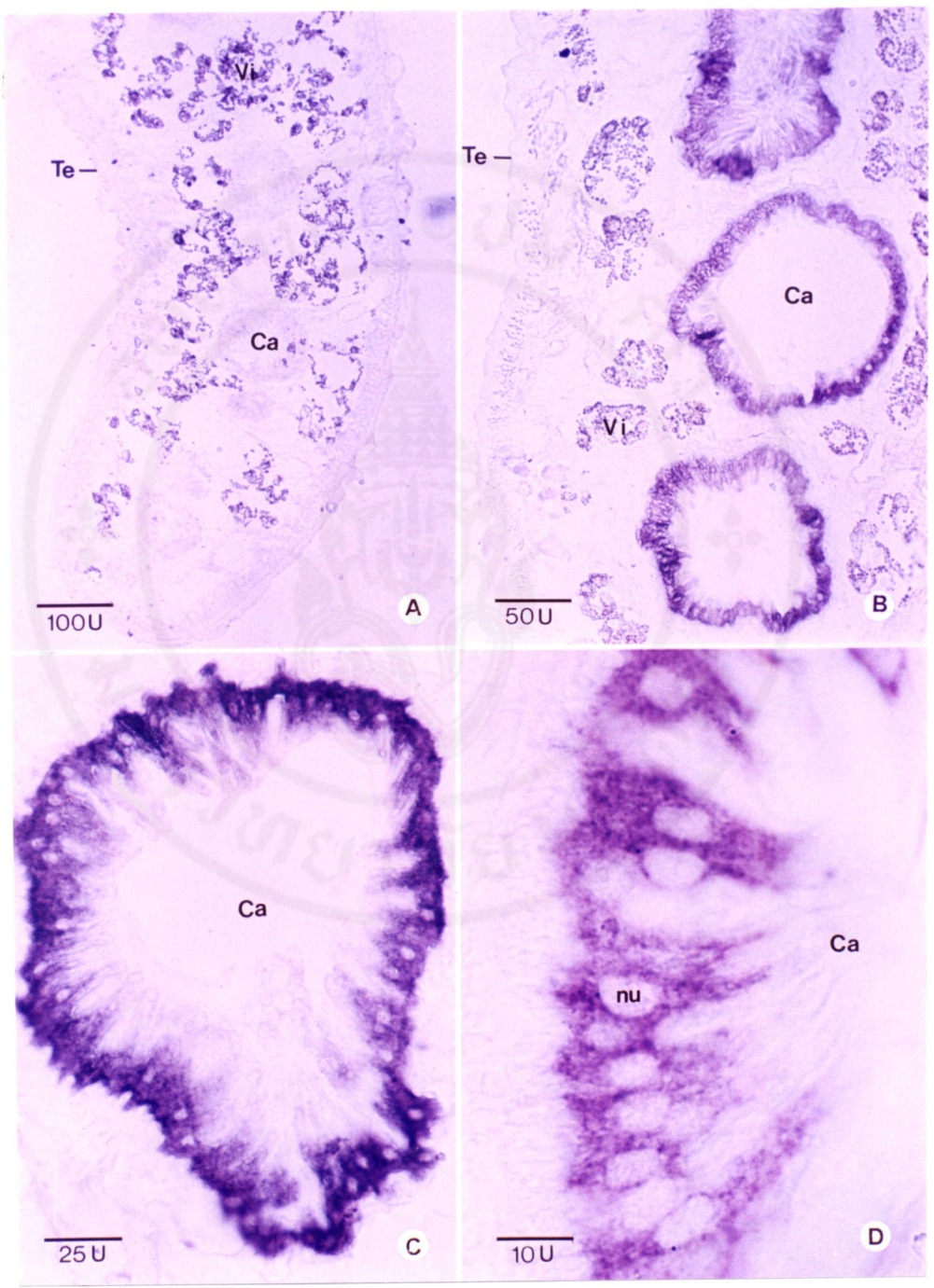


Figure 16. Localization of Cat L mRNA in paraffin sections of the anterior part of adult *F. gigantica* by *in situ* hybridization using DIG-labeled cDNA probe.

A) Cross section stained with the probe showing positive staining in the caecum (Ca), while the pharynx (Ph) and the anterior part of the caecal bifurcation (Cb) are not stained.

B) A high-power micrograph showing positive staining in the caecum (Ca), while the epithelium (Ep) of the pharynx (Ph) is not stained.

C) A high-power micrograph showing positive staining in the posterior part of caecal bifurcation (Cb).

D) Higher magnification of the caecal bifurcation (Cb) in Fig. C showing the transition point (arrows) from tegumental-type to digestive-type epithelium. The inset is higher magnification of the caecal bifurcation (Cb) showing positive staining in the cytoplasm of digestive-type epithelial cell, while tegumental-type epithelial cells are not stained.

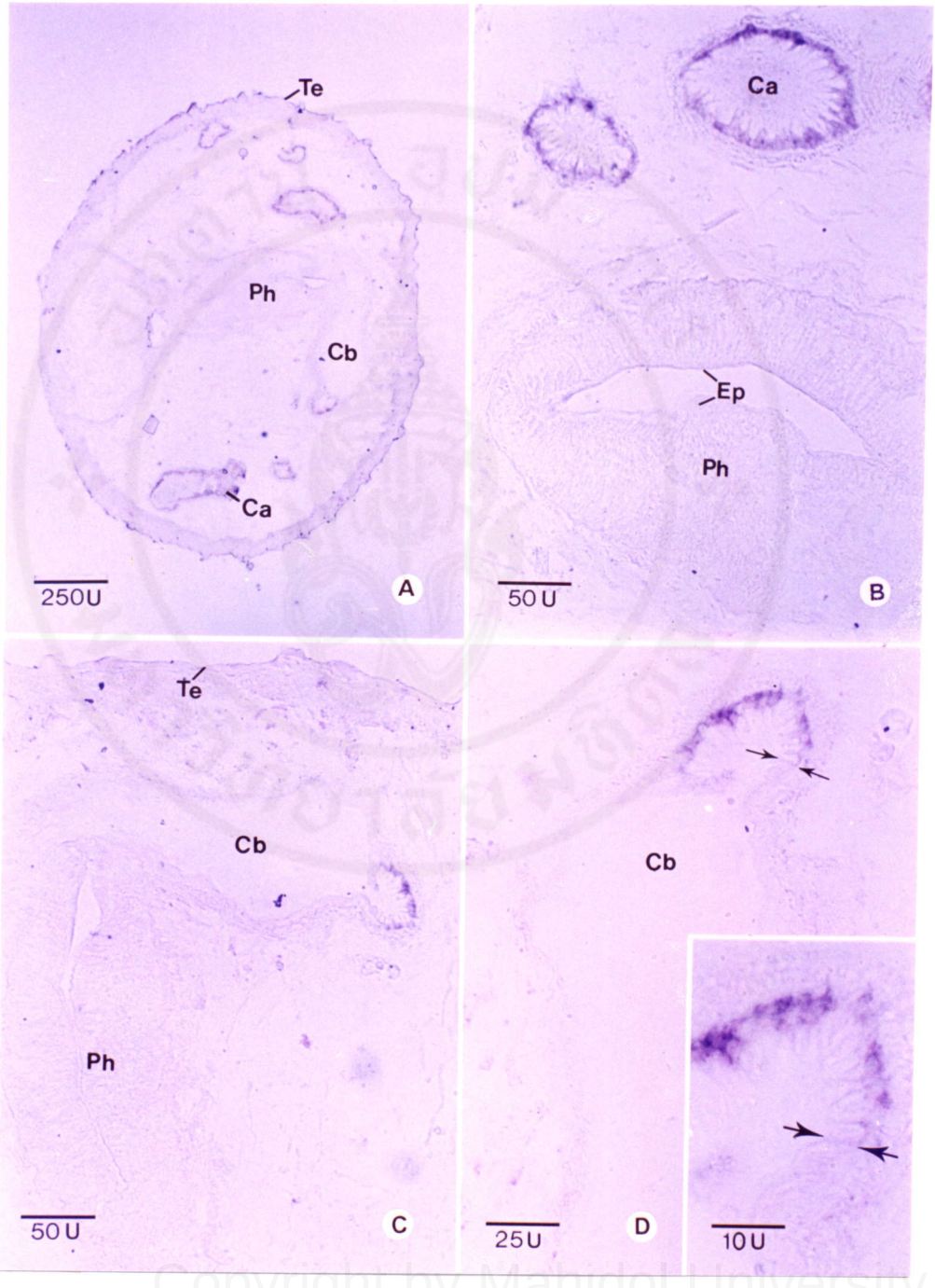


Figure 17. Localization of Cat L mRNA in paraffin sections of the anterior and middle parts of adult *F. gigantica* by *in situ* hybridization using DIG-labeled cDNA probe.

A) Cross section of the middle part of *F. gigantica* showing positive staining in the epithelium of the caecum (Ca), while the testis (Ti) are not stained.

B) Higher magnification of the caecum in Fig. A showing positive staining in the cytoplasm of the caecal epithelial cell (Ca).

C) Cross section of the anterior part of *F. gigantica* showing positive staining in the epithelium of the caecum (Ca), while the uterus (Ut) is not stained.

D) Higher magnification of the adjacent section showing intense staining in the cytoplasm of the caecal epithelial cells (Ca).

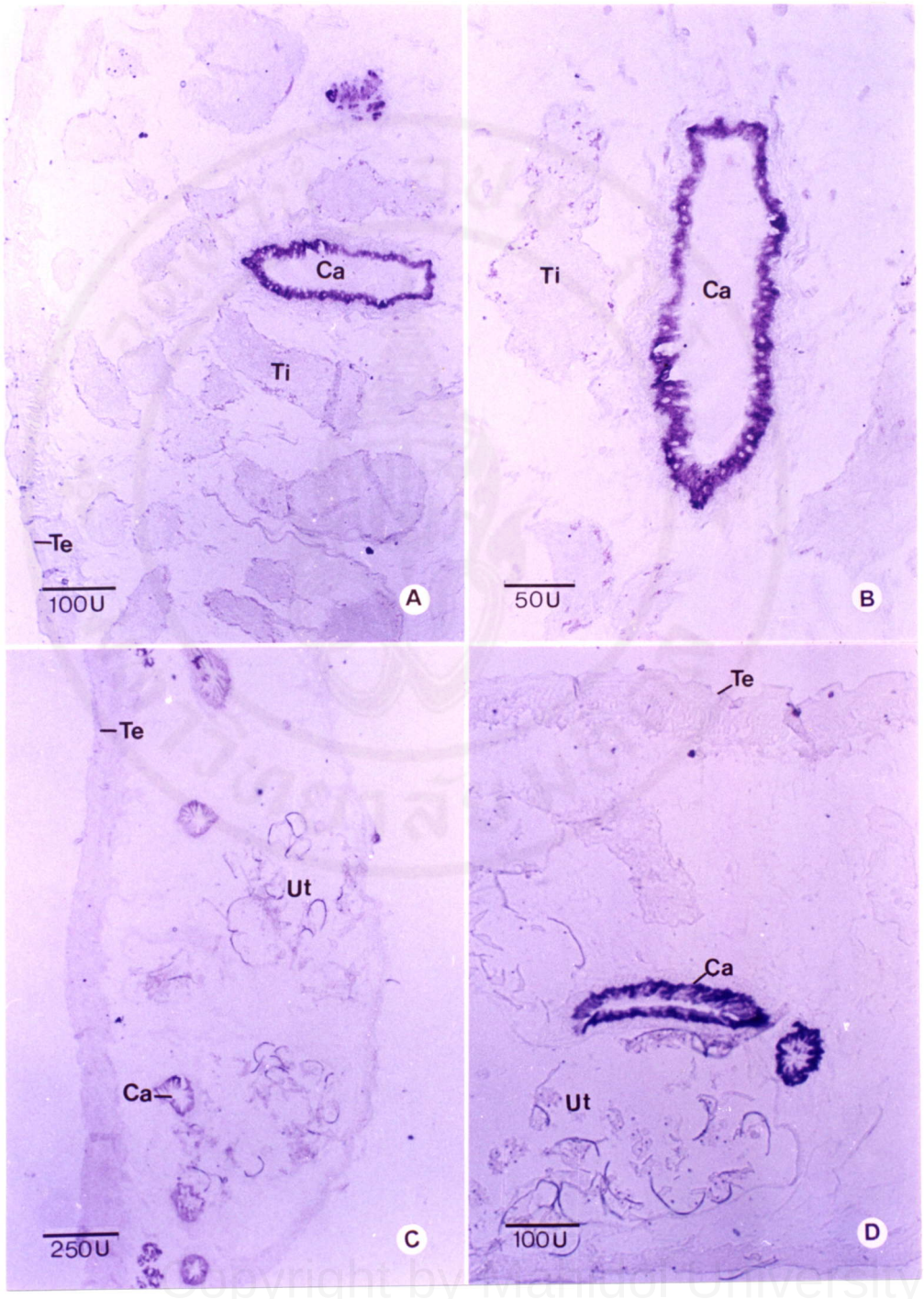


Figure 18. Localization of Cat L mRNA in paraffin sections of the middle part of 50-day-old juvenile *F. gigantica* by *in situ* hybridization using DIG-labeled cDNA probe.

A) Control section stained with hybridization buffer instead of probe showing no positive staining in the caecum (Ca) and tegument (Te).

B,C) Cross sections stained with the probe showing positive staining in the caecum (Ca), while the tegument (Te) is not stained.

D) Higher magnification showing positive staining in the cytoplasm of the caecal epithelial cell (Ca), while the nuclei (nu) of the epithelial cells are not stained.

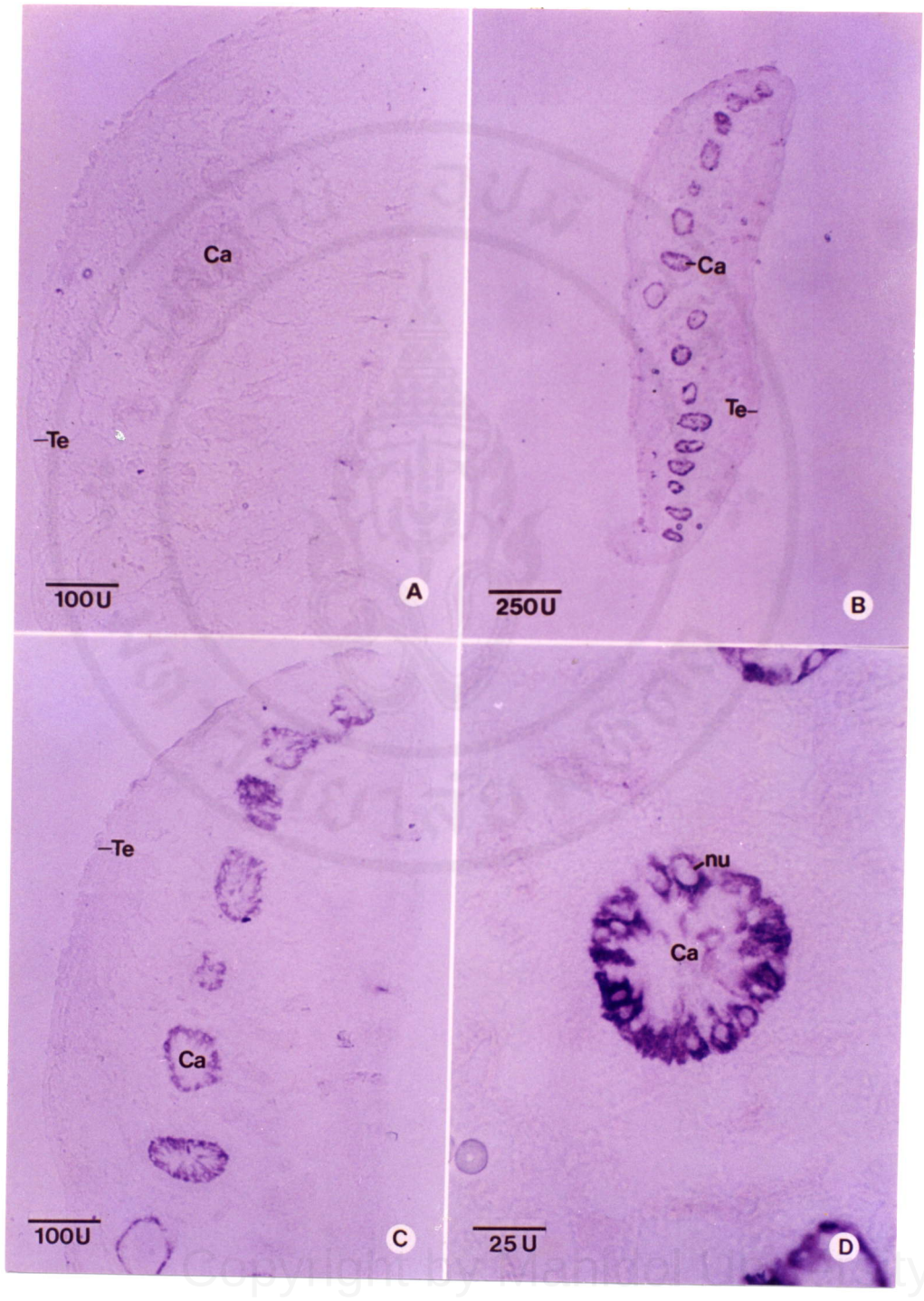


Figure 19. Localization of Cat L mRNA in paraffin sections of the anterior part of 50-day-old *F. gigantica* by *in situ* hybridization using DIG-labeled cDNA probe.

A) A low-power micrograph showing positive staining in the caecum (Ca).

B) Higher magnification showing no positive staining in the epithelium (Ep) of the pharynx (Ph).

C) A low-power micrograph showing positive staining in the caecum (Ca)

D) Higher magnification of the caecal bifurcation (Cb) in Fig. C showing the transition point (arrows) from tegumental-type to digestive-type epithelium. The positive staining is confined to the cytoplasm of the digestive-type epithelial cell, while the tegumental-type epithelial cells are not stained.

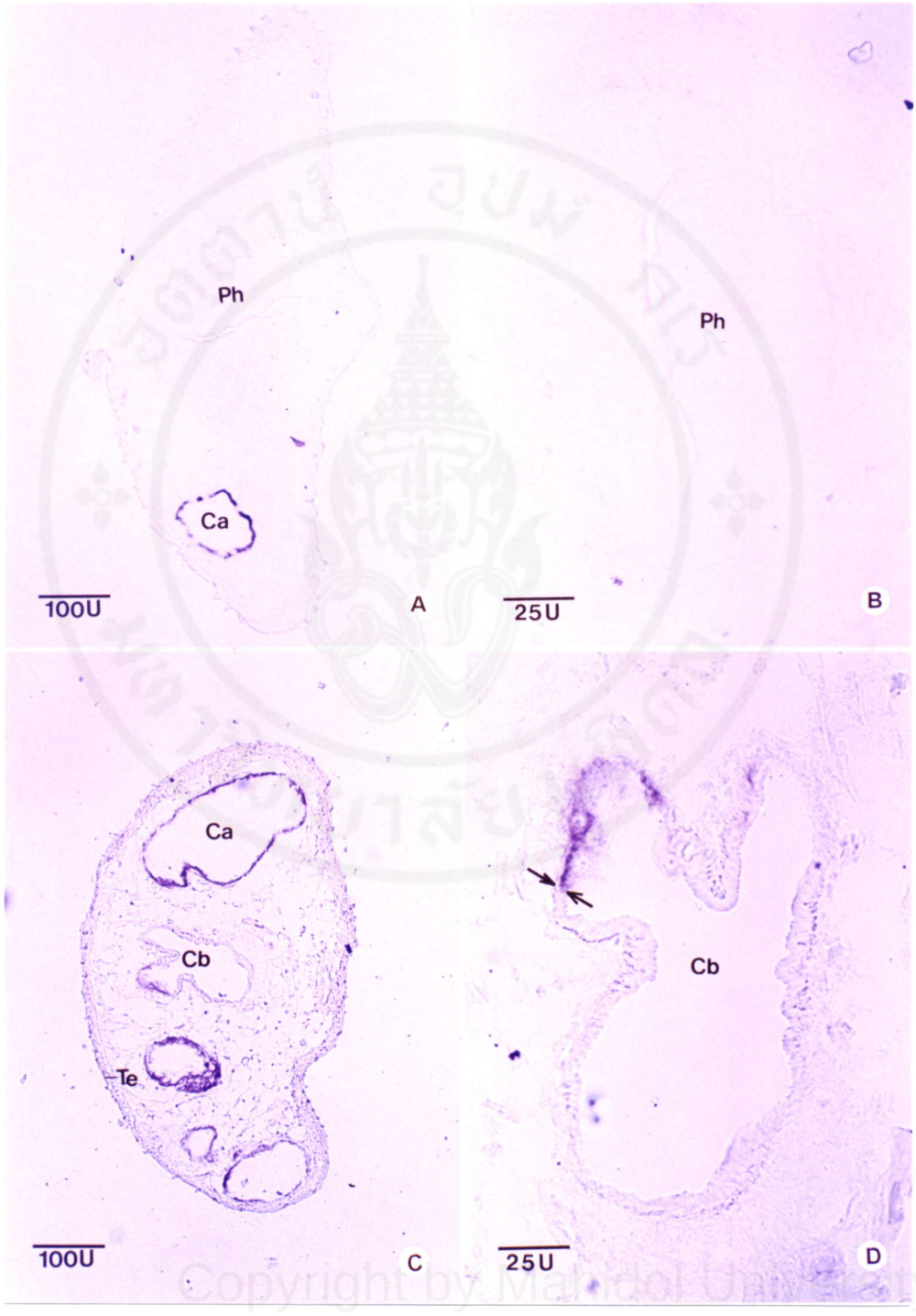


Figure 20. Localization of Cat L mRNA in paraffin sections of the tissue of 50-day-old *F. gigantica* by *in situ* hybridization using DIG-labeled cDNA probe.

A) Cross section of the anterior part of 50-day-old *F. gigantica* showing positive staining in the caecum (Ca). Vs = ventral sucker

B,C) Cross sections through Mehlis' gland (Mg) showing positive staining only in the caecum (Ca).

D) Cross section of the posterior part of the parasite showing positive staining in the caecum (Ca).

E) Higher magnification of the caecum (Ca) in Fig. D showing intense staining in the caecal epithelium. Te = tegument

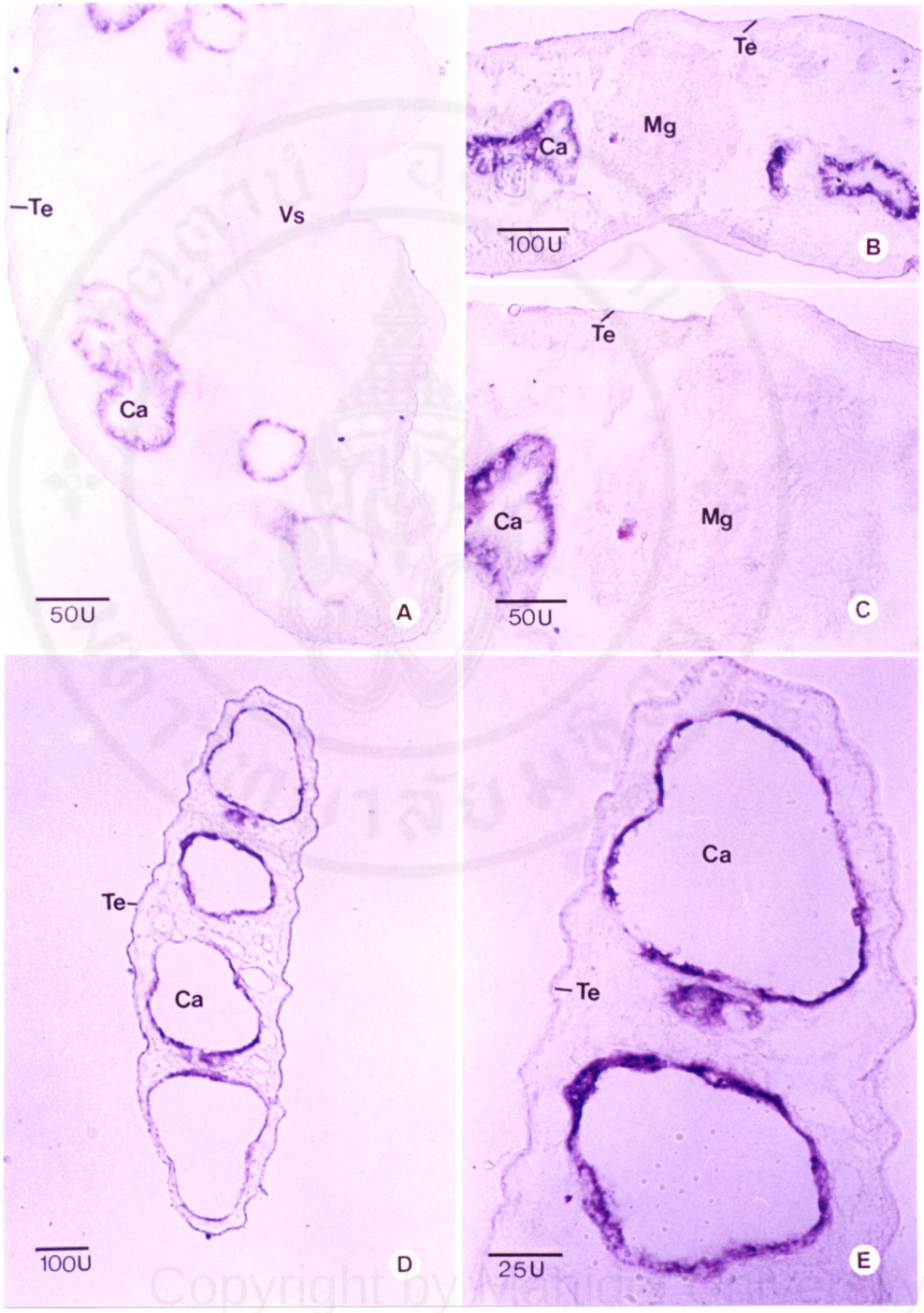


Figure 21. Localization of Cat L mRNA in a paraffin section of the metacercaria of adult *F. gigantica* by *in situ* hybridization using DIG-labeled cDNA probe. Positive staining can not be detected in any part of the tissue section. Ca = caecum, Te = tegument

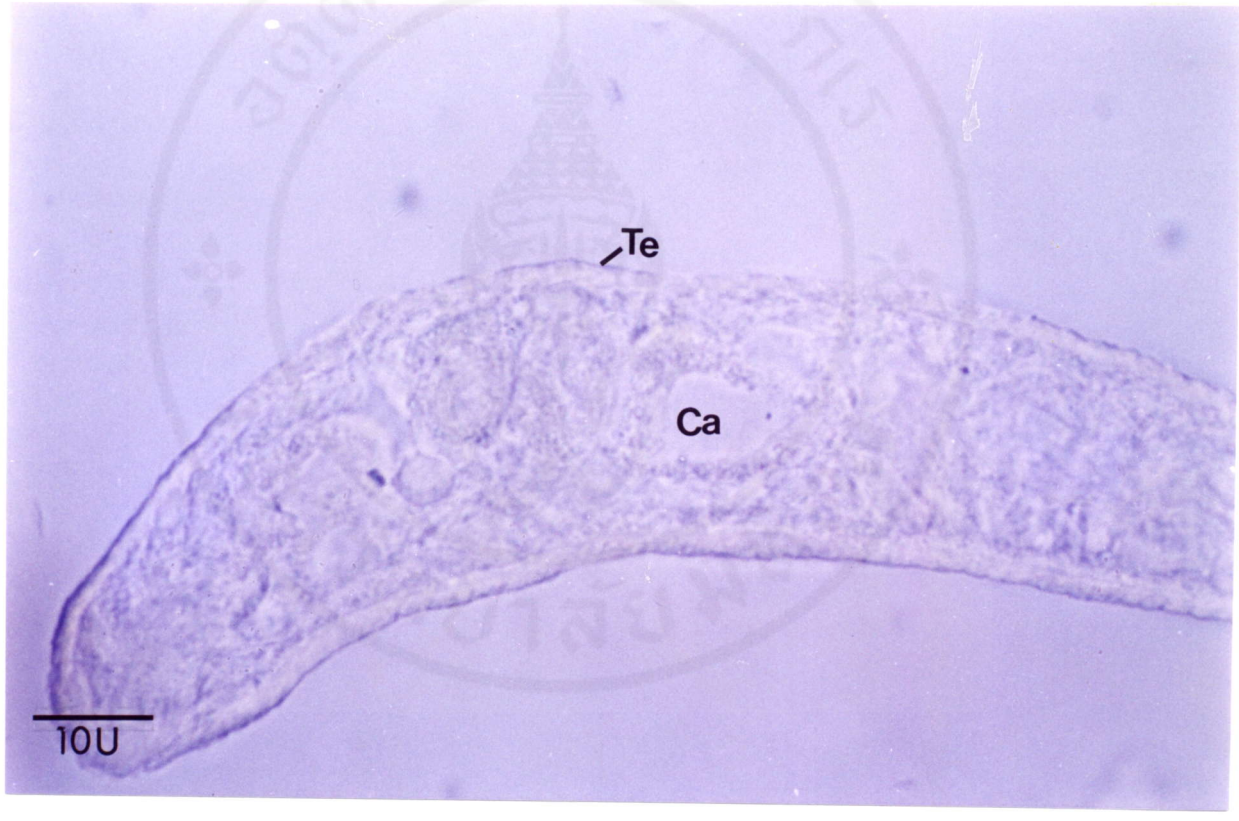


Figure 22. Frozen sections of the posterior part of the adult *F. gigantica* stained by immunofluorescence technique, using 2D9 MoAb (against 26-27 kD ES antigen) as primary antibody and FITC-conjugated goat anti-mouse IgG as secondary antibody.

A) Control section showing no positive staining, but yellowish autofluorescence appear in the vitelline glands (Vi).

B) Cross section stained with the primary antibody showing positive staining (arrows) in the central part of the caecal lumen.

C,D) Higher magnification of the caecum in Fig. B showing moderate and even fluorescence in the cytoplasm of the caecal epithelial cells (Ep), while intense fluorescence occur at the content of the caecal lumen (arrows).

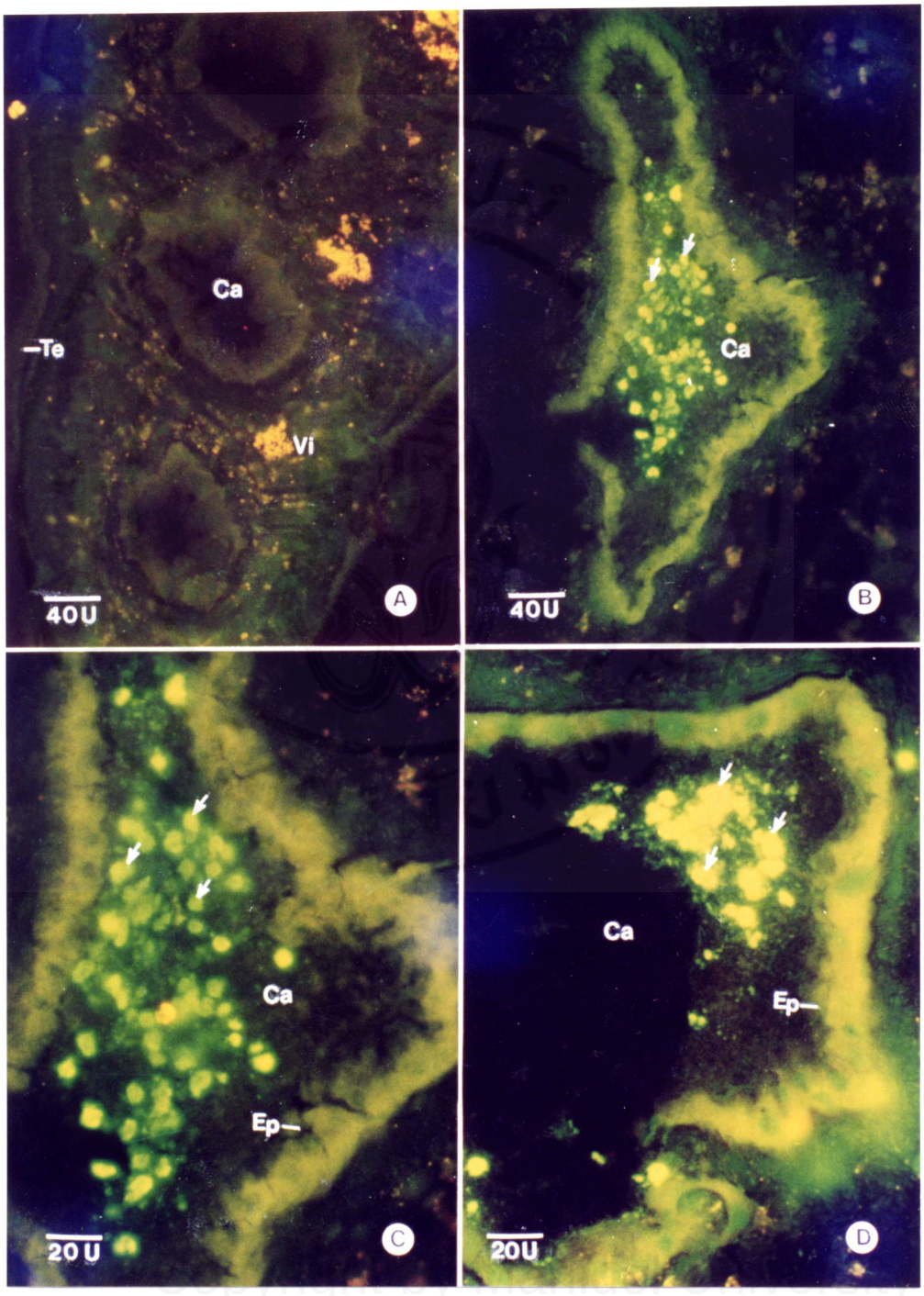


Figure. 23. Frozen sections of the cross section of the posterior part of the adult *F. gigantica* stained by immunofluorescence technique.

A,B) Low and high-power micrographs of Sections stained with normal sheep serum as primary antibody and FITC-conjugated rabbit anti-sheep IgG as secondary antibody. Positive fluorescence staining is not seen over any part of the organ system, but non-specific staining is seen at the outer surface of the tegument (Te) and spine (Sp).

C,D) The sections were stained with normal mouse serum as primary antibody and FITC-conjugated goat anti-mouse IgG as secondary antibody. Positive fluorescence staining is not seen in the caecum (Ca), testis (Ti), and vitelline gland (Vi).

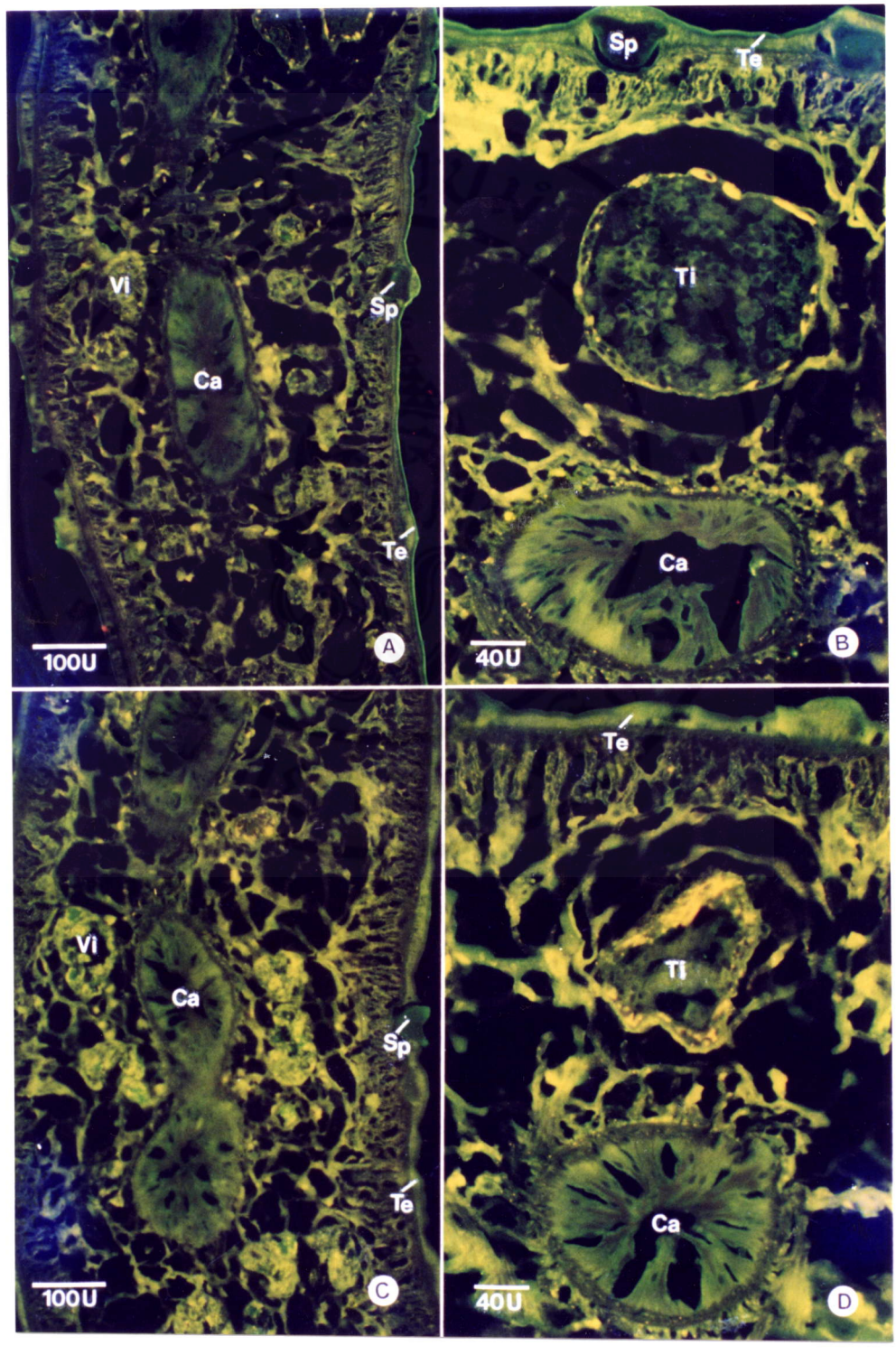

Vi
Sp
Ca
Te
100U
A
Sp
Te
Ti
Ca
40U
B
Vi
Ca
Sp
Te
100U
C
Te
Ti
Ca
40U
D

Figure 24. Frozen sections of the posterior part of the adult *F. gigantica* stained by immunofluorescence technique, using PoAb mouse anti-Cat L *F. gigantica* as primary antibody and FITC-conjugated goat anti-mouse IgG as secondary antibody.

A,B) Low-power micrographs showing the positive staining in the cytoplasm of the caecal epithelial cell (Ca) and non-specific fluorescence staining at outer surface of tegument (Te) and spine (Sp). No staining can be observed in the vitelline gland (Vi) and testis (Ti).

C) A high-power micrograph showing strong fluorescence in the cytoplasm of the caecal epithelial cell (Ep).

D) Higher magnification showing intense staining in the cytoplasm of the epithelial cell (Ep) and accumulation of the granules (arrows) in the apical cytoplasm, while nuclei (nu) are negatively stained.

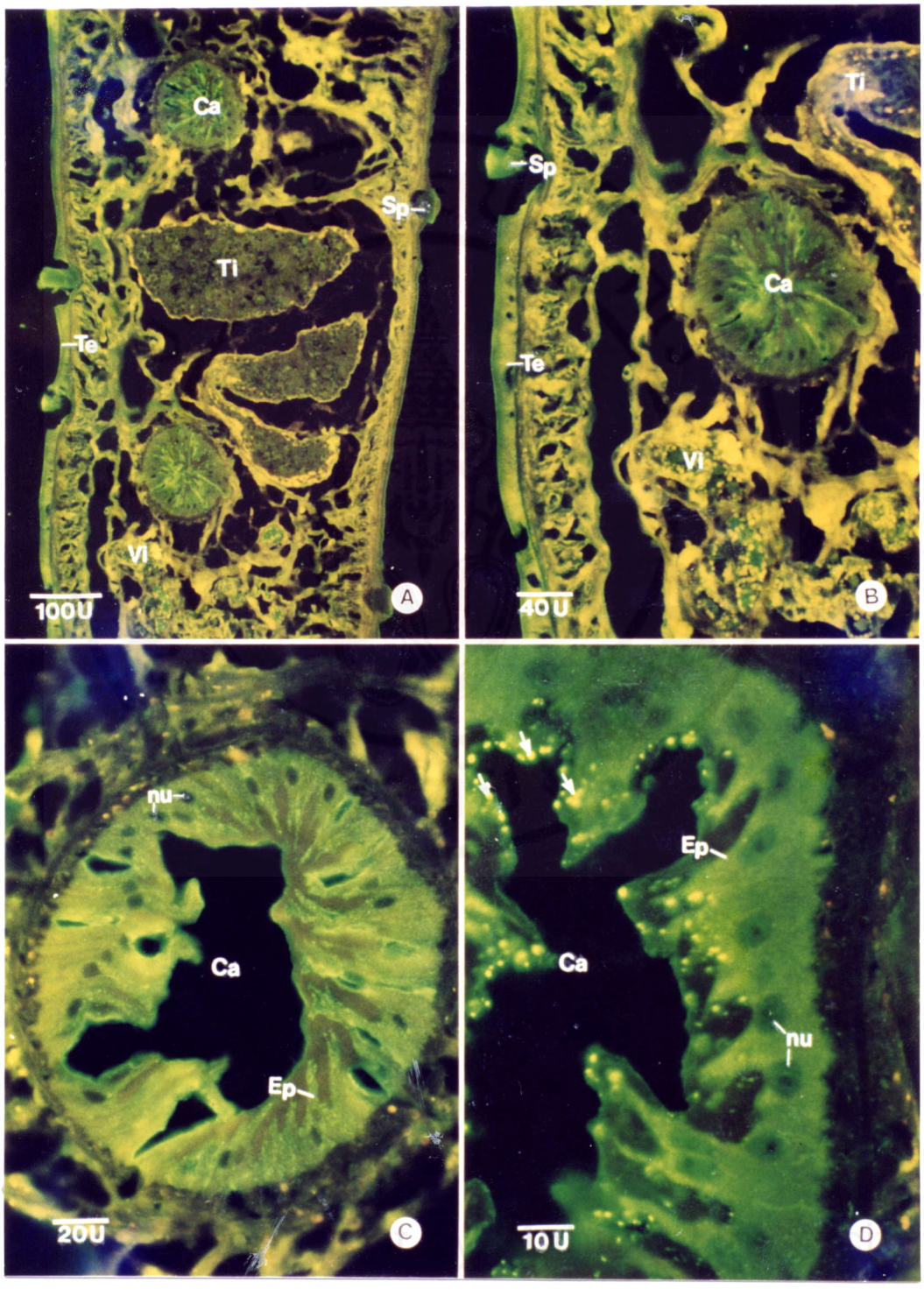

Ca
Sp
Ti
Te
Vl
100 U
A
Ti
Sp
Ca
Te
Vl
40 U
B
nu
Ca
Ep
20 U
C
Ep
Ca
nu
10 U
D

Figure 25. Frozen sections of the posterior part of the adult *F. gigantica* stained by immunofluorescence technique, using PoAb sheep anti-Cat L *F. hepatica* as primary antibody and FITC-conjugated rabbit anti-sheep IgG as secondary antibody.

A) A low-power micrograph showing the positive staining in the caecum (Ca).

B) Higher magnification showing non-specific fluorescence staining at the outer surface of tegument (Te) and spine (Sp), and negative staining in the vitelline gland (Vi).

C) A high-power micrograph showing positive fluorescence in the cytoplasm of the caecal epithelial cell (Ep).

D) Higher magnification showing that intense staining in the cytoplasm of the caecal epithelial cell (Ep) appears in form of very fine granules (arrows), while nuclei (nu) are negatively stained.

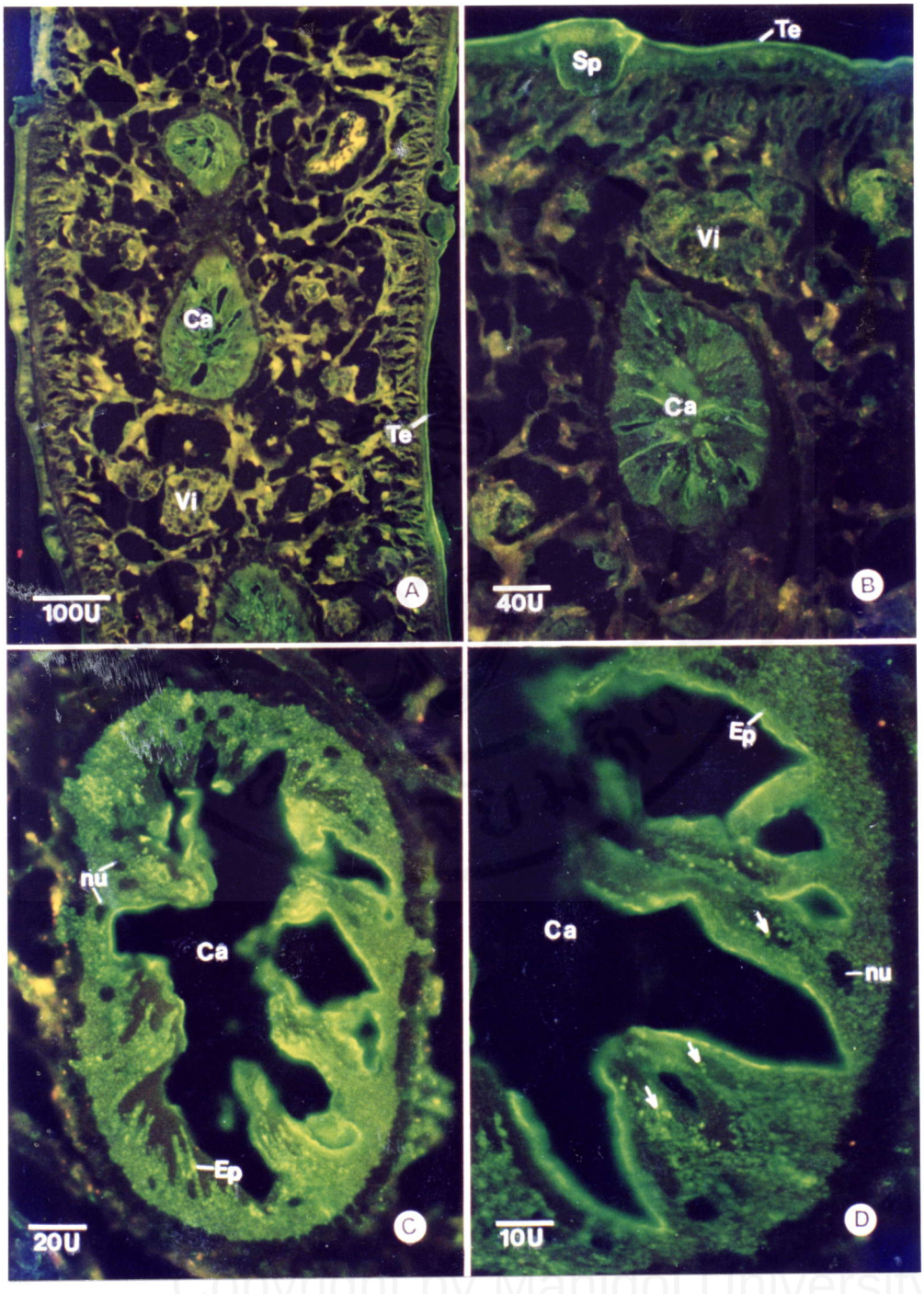


Figure 26. Frozen sections of the anterior part of the adult *F. gigantica* stained by immunofluorescence technique, using PoAb mouse anti-Cat L *F. gigantica* as primary antibody and FITC-conjugated goat anti-mouse IgG as secondary antibody

A) Cross section of the pharynx (Ph) and buccal tube (Bt) showing only weak non-specific fluorescence staining at the outer surface of the epithelium (Ep).

B) A low-power micrograph of the part distal to the esophagus showing strong fluorescence in the caecum (Ca) and digestive-type epithelium at the caecal bifurcation (Cb).

C) Higher magnification of the caecal bifurcation (Cb) in Fig. B showing the transition point (arrows) from the tegumental-type to digestive-type epithelium.

D) An enlarged view of the epithelium in Fig C showing strong fluorescence staining in the cytoplasm of digestive-type epithelial cells, while non-specific staining is seen in the outer surface of the tegumental-type epithelium.

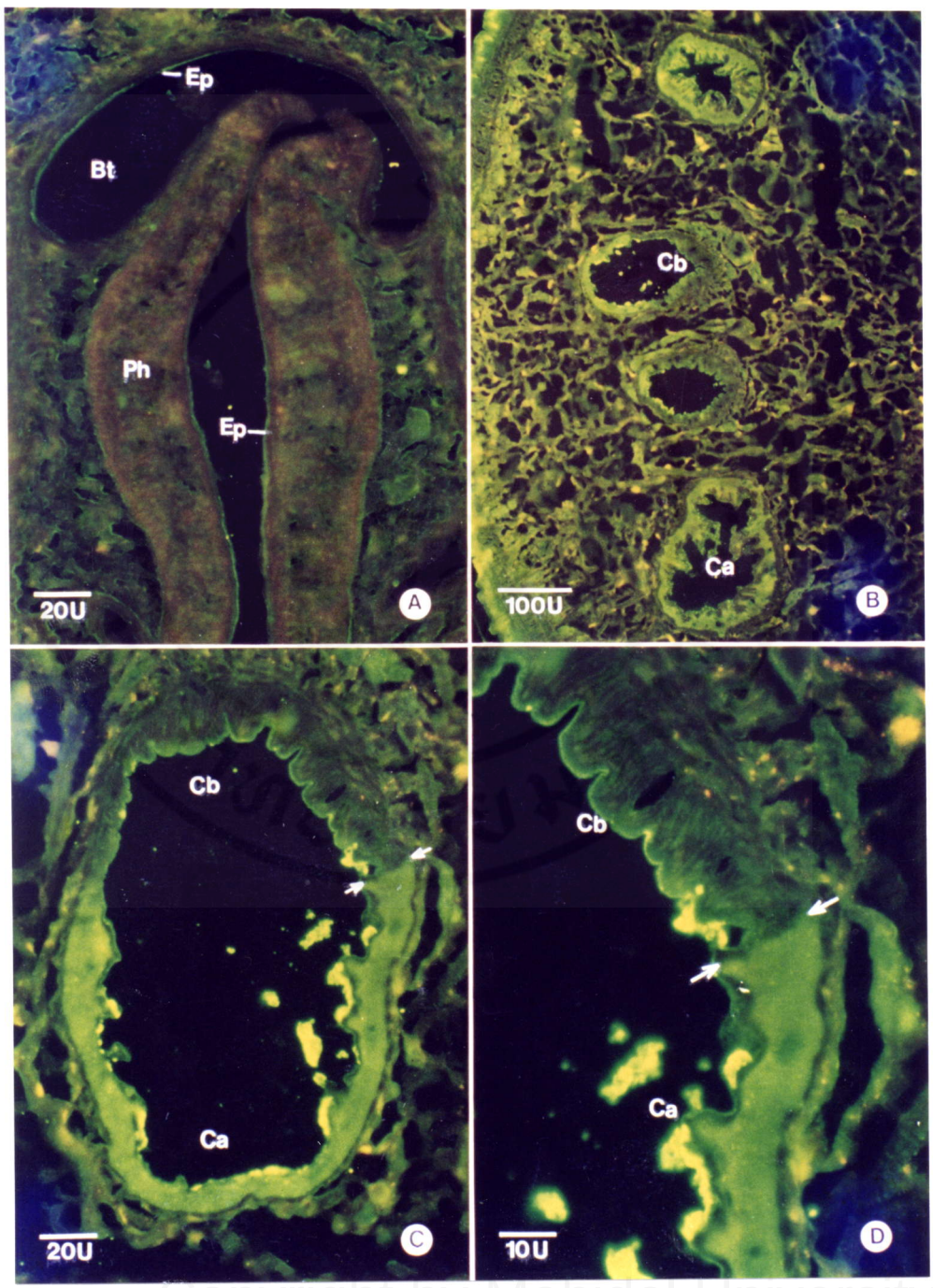


Figure. 27. Frozen sections of the anterior part of the adult *F. gigantica* stained by immunofluorescence technique, using PoAb sheep anti-Cat L *F. hepatica* as primary antibody and FITC-conjugated rabbit anti-sheep IgG as secondary antibody

A) A cross section of the pharynx (Ph) showing weak non specific staining at the outer surface of the epithelium (Ep).

B) A low-power micrograph of the part distal to the esophagus showing strong fluorescence in the caecum (Ca).

C) Higher magnification of the caecal bifurcation (Cb) in Fig. B showing the transition point (arrows) from the tegumental-type to digestive-type epithelium.

D) An enlarged view of the epithelium in Fig. C showing strong fluorescence staining in the cytoplasm of digestive-type epithelial cells, while non-specific fluorescence staining is seen at the outer surface of the tegumental-type epithelium.

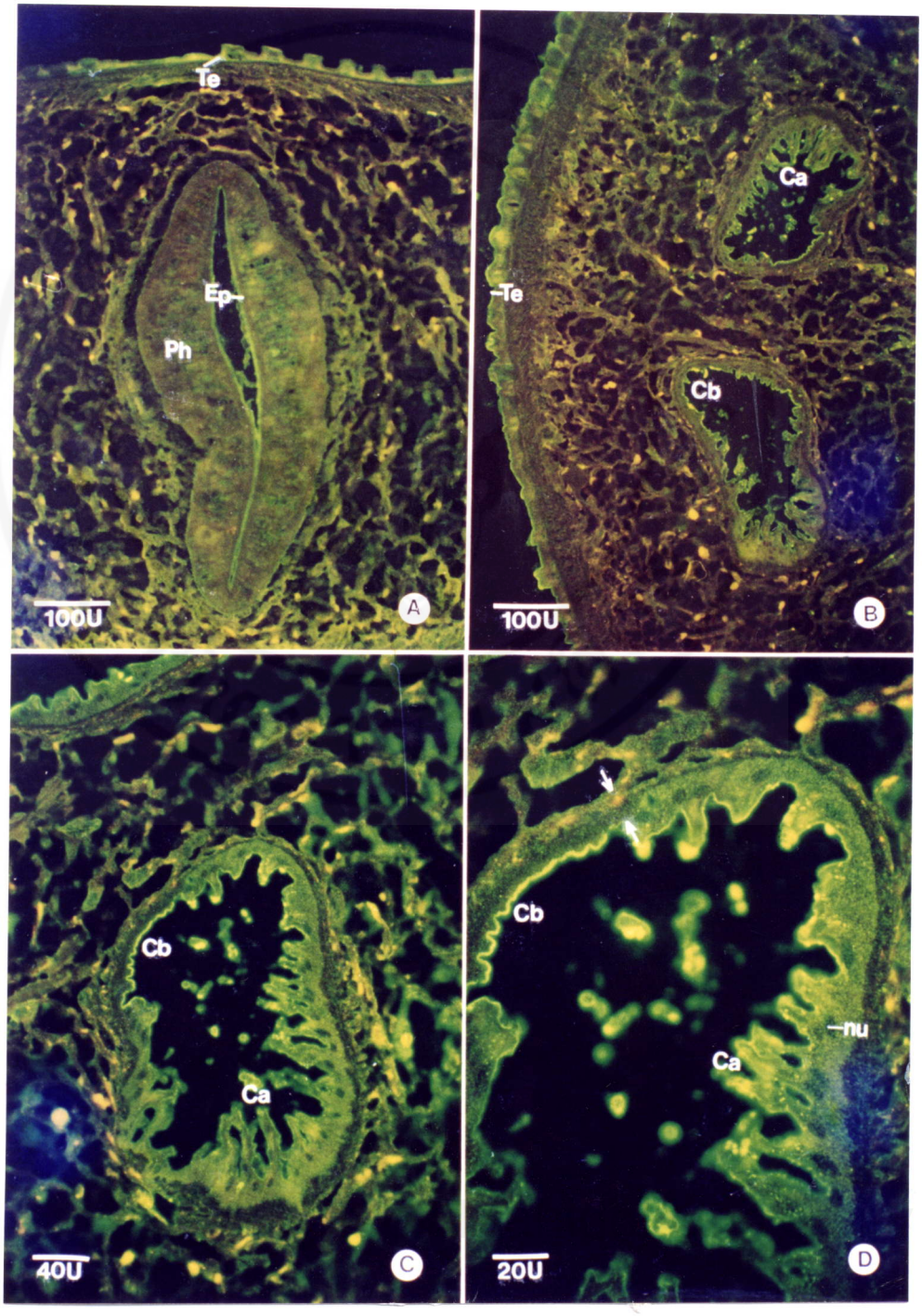

Te
Ep
Ph
100U
A
Ca
Te
Cb
100U
B
Cb
Ca
40U
C
Cb
nu
Ca
20U
D

Figure 28. Frozen sections of the middle part of the 50-day old juvenile *F. gigantica* stained by immunofluorescence technique, using PoAb mouse anti-Cat L *F. gigantica* as primary antibody and FITC-conjugated goat anti-mouse IgG as secondary antibody.

A) A low-power micrograph of a control section stained with normal mouse serum showing no fluorescence staining, but non-specific staining appears at the outer surface of the tegument (Te).

B) A low-power micrograph of an adjacent section stained with the primary antibody showing positive staining in the caecum (Ca).

C) Higher magnification of the caecum (Ca) in Fig. B. showing positive fluorescence staining in the cytoplasm of the caecal epithelial cell (Ep)

D) Higher magnification of the caecum in Fig. C showing positive staining in the cytoplasm of the caecal epithelial cell (Ep); intense staining appears at the apical surface of the epithelium (arrows).

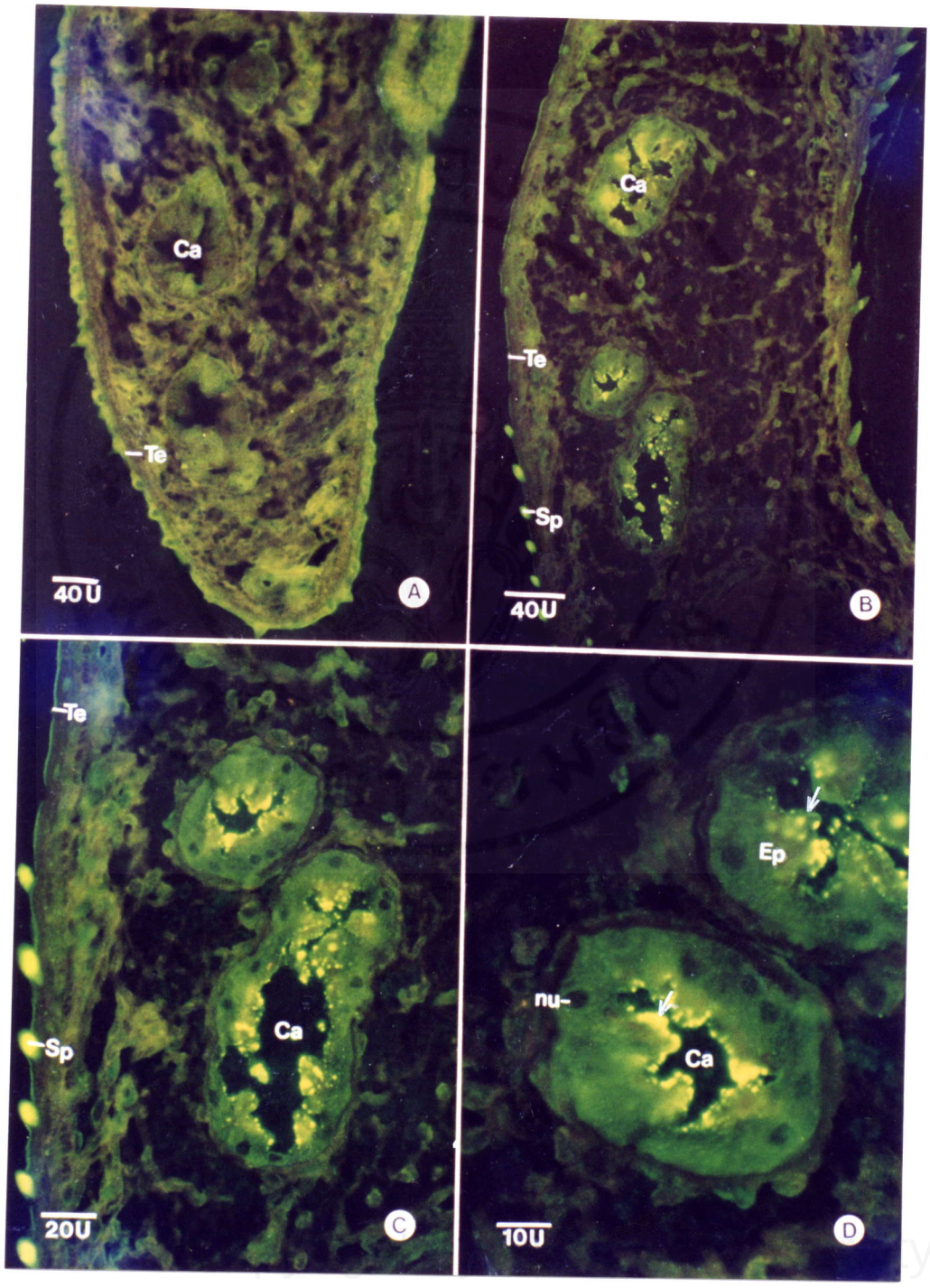

Ca
Te
40 U
A
Ca
Te
Sp
40U
B
Te
Sp
Ca
20U
C
Ep
nu
Ca
10U
D

Figure 29. Frozen sections of the middle part of the adult *F. gigantica* stained by immunofluorescence technique, using PoAb sheep anti-Cat L *F. hepatica* as primary antibody and FITC-conjugated rabbit anti sheep IgG as secondary antibody.

A) A control section showing no fluorescence staining in the caecum (Ca).

B) A low-power micrograph showing the positive fluorescence staining in the caecum (Ca) and non-specific fluorescence staining at the outer surface of the tegument (Te).

C) Higher magnification of the caecum (Ca) in Fig. B showing positive fluorescence staining in the cytoplasm of the caecal epithelial cell (Ep).

E) Higher magnification of the caecum in Fig. C showing that intense staining in the cytoplasm of caecal epithelial cell (Ep) appears in form of very fine granule (arrows), while nuclei (nu) are negatively stained.

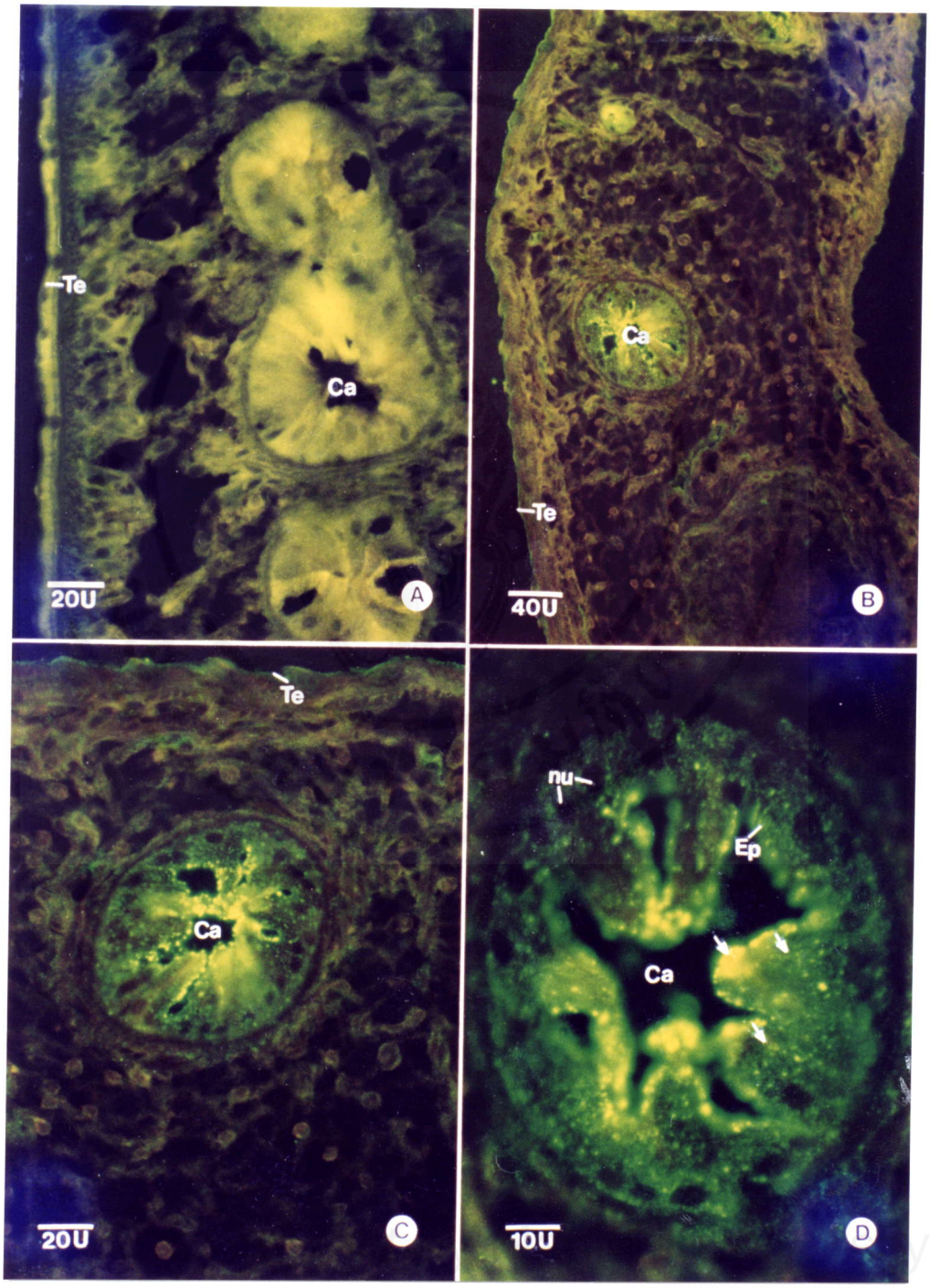
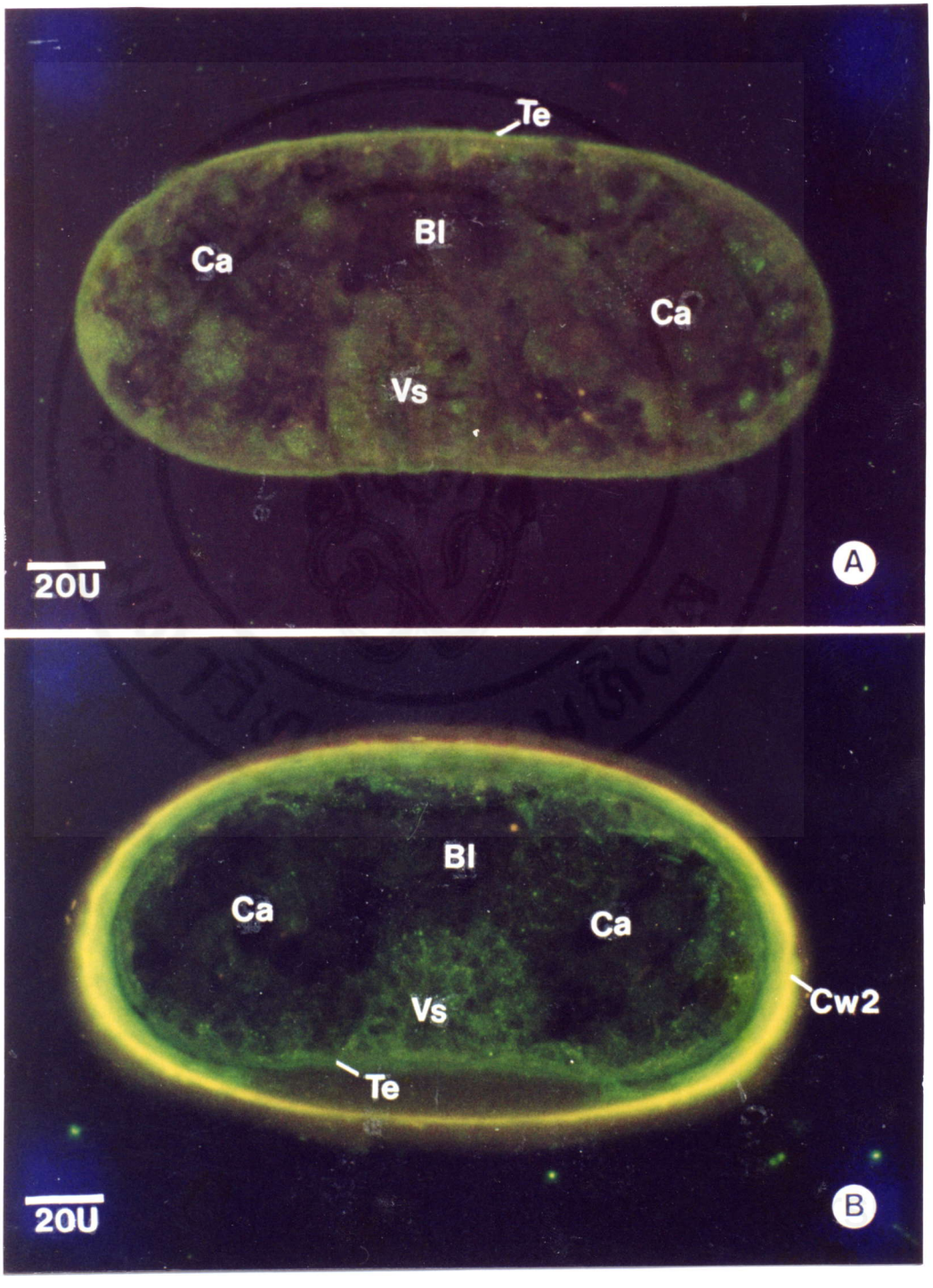


Figure 30. Frozen sections of the middle part of the metacercariae of *F. gigantica* stained by immunofluorescence technique.

A) A cross section stained with PoAb mouse anti-Cat L *F. gigantica* as primary antibody and FITC-conjugated goat anti-mouse IgG as secondary antibody . Positive fluorescence staining is not seen in the caecum (Ca), ventral sucker (Vs), bladder (Bl) and tegument (Te).

B) A cross section stained with PoAb sheep anti-Cat L *F. hepatica* as primary antibody and FITC-conjugated rabbit anti-sheep IgG as secondary antibody showing no positive staining in the caecum (Ca), ventral sucker (Vs), bladder (Bl) and tegument (Te). Non-specific fluorescence staining appears at the protective cyst of the metacercaria (Cw2).


Te
Bl
Ca
Ca
Vs
20U
A
Bl
Ca
Ca
Cw2
Vs
Te
20U
B

CHAPTER VI

DISCUSSION

1. Gross Anatomy and Histology of the Digestive Tract of *Fasciola gigantica*

Gross morphology of the digestive tract of adult *F. gigantica* has been reported earlier by Meepool (1994) (33). It is composed of the oral sucker, buccal tube, pharynx, esophagus and caeca. The oral sucker is connected with the pharynx by the buccal tube. The caecum is continuous with the esophagus and bifurcated into left and right main caeca which give off numerous branches. In several reports, the oral sucker of most digenean parasites are located at the anterior tip of body, except in *Bucephaloides gracilescens*, the oral sucker is ventrally located and is not seen at the anterior end as in the digenea (35). In *F. hepatica* the oral sucker and ventral sucker are almost equal in size, with the ventral sucker appears slightly larger. On the other hand for *F. gigantica*, the ventral sucker is larger than the oral sucker with the ratio of these is being 3:2 (80). The pharynx of most digenean parasite has the same histological pattern, as found in *Haematoloeclus medioplexus*, *Haplometra cylindracea*, *Opisthioglyphe ranae*, *Opistherchis viverrini* and *Fasciola hepatica* (81). Moreover, in *Diplodiscus subclavatus*, *Gorgoderina vitelliloba* and *Gorgodera cygnoides*, there is no true pharynx; the wall of the esophagus contains thick muscular

layer and appears to compensate for its absence (81). The secretory cells are found around the wall of oral sucker and pharynx of *H. cylindracea* and *O. ranae* (81). By contrast, in the present study, the secretory cells were not found in the oral sucker and pharynx of *F. gigantica*.

It is observed in the present study that the caecum of adult *F. gigantica* extends towards the posterior end of the body and has numerous branches This extensive branching occur since the adult of *F. gigantica* is comparatively large in size and needs a lot of food for living. This parasite has no the circulatory system, and it is likely that the extensive branching of digestive tracts enable food materials to be delivered directly to the body tissues. Hence, the digestive system is well developed and the highly branched caeca are distributed to all parts of the body instead of capillary network found in higher animals It has already been pointed out by many researchers that the branching of caeca of *F. gigantica* are much more complicated than those of *F. hepatica* (81). This highly branched form of caeca appears to be peculiar to certain member of the family Fasciolidae, in contrast to the other digenea which have the simple unbranched caeca. In *Bucephaloides gracilescens*, the caecum is sac-like structure that does not bifurcate into two branches (35). In the blood fluke, *Schistosoma mansoni*, the caecum is unusual in that the paired caeca rejoin about the middle of the body and become continuous single winding tube, ending blindly at posterior tip of the body (35).

In the present study, 50-day-old juvenile *F. gigantica* were obtained from the liver of mice at 50 days after the oral infection with metacercariae. This parasite is in immature form, because it has not reached sexual maturity as judged by the development of reproductive organs (82). The fully mature parasite is distinguished

by the appearance of the testis and ovary, and they can produce normal eggs which are liberated into the bile duct and the intestine, which are finally found in the faeces of the hosts (45). In comparison to the reproductive system, the digestive tract of juvenile *F. gigantica* is fairly well developed and exhibit most features similar to that of the adult *F. gigantica*; however, the caeca of the adult stage have more extensive branching when compared to the juvenile stage.

In metacercaria, the digestive tract is composed of the oral sucker, pharynx and pair of lobulated caeca which curve round the ventral sucker and are situated in the posterior half of the body. The two caeca are located more ventrally and near the midline and terminate blindly close to the posterior end of the parasite. In comparison to adult and juvenile stages, the caeca of metacercarial stage are unbranched. Many investigators suggested that in the metacercarial stage gut is still non functional and most energy required is derived from endogenous sources, particulary glycogen (83). This may explain why the digestive tract of metacercaria of *F. gigantica* appear rather rudimentary. The gross development of the digestive tract during the growth of *F. hepatica* in the mouse has been described by Bennett (1975). Initially in the NEJ, the caeca are short and start to show signs of elongation in the first day following excystment. The lateral branches begin to develop in day 3 post-infection. There are 13 club-shaped branches on each side of the body at day 8 post-infection. Secondary and tertiary branches are present by day 11 post-infection (46). The ramification of caeca begins after excystment and during migration in the abdominal cavity, and when the parasite penetrate through the capsule of the liver of the host the caecum becomes more highly branched. This suggests that the number of branches of the caeca are

increased and the digestive tract become functional only after the penetration and migration start and the tract develop quite rapidly.

In the present study, the epithelium of the digestive tracts of 50-day-old *F. gigantica* is similar to that of the adult stage. It consists of two types: the tegumental-type which is a syncytium essentially similar to the tegument, and the digestive-type which is composed of an array of tall columnar to cuboidal digestive cells. The proximal parts of the digestive tract, including the oral sucker, buccal tube, pharynx, esophagus, and part of the tract anterior to the caecal bifurcation, are lined by tegumental-type epithelium. By contrast, the caecum is lined by the digestive-type epithelium. Many investigators have observed that the oral suckers of *Schistosoma mansoni*, *Schistosoma japonicum*, *Schistosoma mekongi* and *Schistosomatium douthitti* are lined by the tegument which closely resembles that covering the general body surface (48, 49, 84). In the pharynx and esophagus, the tegument becomes deeply invaginated with long narrow folds of the basal plasma membrane. In the present study, the tegumental-type epithelium is also distinguished by numerous basal infoldings which extend towards the apical surface similar to the tegument that covers the body surface of the parasite (85). This observation suggests that the epithelium lining the proximal part of the digestive tract and the tegument might be derived from the same primary germ layer (outer germ layer, or ectoderm) in the embryonic period of development. In *Megalodiscus temperatus* and *S. mansoni*, the transition point from tegument to caeca is abruptly marked by a long junctional complex, whose type is still not clearly stdudied. In our study, the transition point from tegumental-type to digestive-type occurs at the caecal bifurcation where epithelial at both side of the junction show markedly different characteristics. The junctional complex between the

tegumental-type to digestive-type epithelium has yet to be studied by transmission electron microscopy.

It was found that the digenean caeca appears to be syncytial in most species with the quite definite exception in *F. hepatica* in which the layer is truly cellular (34). By contrast, *H. medioplexus* has intermediate feature in which septate desmosomes are observed at the apex of adjacent cells of the caeca, but there are no distinct lateral membranes separating the cells (81). Caecal epithelial cell in digenea is classcified into two distinct categories: 1) the epithelial cells which are irregular in height and shape with microvilli that vary in length from 1-15 μm (*H. medioplexus*, *H. cylindracea*, *O. ranae*, *F. hepatica and S. mansoni*); and 2) the epithelial cells which have regular shape and height with microvilli organized into a definite striated border 10-20 μm high (*D. subclavatus*, *G. vitelloba* and *G. cygnoides*) (81). Dawes (1962) showed that the most obvious and remarkable feature of the caecal epithelium of *F. hepatica* is that shorter cells line caeca which contain much food, while taller cell occur in the other parts of the caeca in which there little or no food (45). The short cells are most commonly seen in the large primary and secondary branches of the caeca and they measure about 0.01-0.02 mm. from the basal membrane to the free margin The tall cells are more often seen in the smaller caeca, and are about 0.04-0.05 mm in height. In the present study, the caecal epithelial cells of *F. gigantica* are similar to the first category of cells in the digenean parasites and in *F. hepatica* as reported by Dawes (1962). The digestive-type epithelium is composed of tall columnar or cuboidal cells with round basally-located euchromatic nuclei. The cells have prominent nuclei and their apical surface possess numerous stereocilia. The variation in shape and height of the caecal epithelium may depend on stretching of

cells by the amount of food in the caecum. The wall of the caeca which contains transverse and longitudinal muscles may also modify the height of the epithelium by their contraction and relaxation. Usually, the caecal epithelial cells of 50-day-old *F. gigantica* are most commonly seen to be cuboidal in shape. This suggests that for most of the time the juvenile parasite is very active, penetrating and digesting the host tissue. Hence, the caeca always have a large amount of food in the lumen that causes the stretching of the epithelium.

In the present study, the digestive-type epithelial cells of adult and juveniles *F. gigantica* could be classified into 3 types according to their staining properties and related height. type-1, type-2 and type-3, whose cytoplasm are densely, moderately and lightly stained by basic dyes, and the quantity of zymogen granules are decreasing in order, as well as height. All 3 types of epithelial cells are found in the same area, but the number of each type depends on the amount of food present in the caecal lumen. This indicates that type-1 cell may be in the synthetic phase that produces and stores a lot of granules of digestive enzyme in the cytoplasm, while small amount of food is present in the caecal lumen. On the other hand, type-2 and 3 cells may be in secretory and digestive phases, in which the zymogen granules are exocytosed and the enzyme released for the digestion of the increased food material in the caecal lumen. Type-2 epithelial cell has histological structures between type-1 and 3. This cell type may be in early secretory phase, while type-3 cells may be in late secretory or digestive phase. Hence, it is likely that all 3 types of cells may belong to one group but function in different phases, and that all of them can act both as the digestive as well as absorptive cells.

Meepool (1994) reported that the epithelial cells of adult *F. gigantica* were classified into type-I and type-II epithelial cells (33). The type-I cells are tall columnar with slim branches of the apical surface, each of which has long microvilli and no secretory granules are released from apical surface. The cytoplasm is densely stained with methylene blue. The type-II cells are shorter and appear paler than the type-I epithelial cells. The cytoplasm of type-II cells show dilated RER especially in the apical branches. A lot of zymogen granules are released from the apical surface of type-II epithelial cell. Robinson and Threadgold (1975) have found that the caecum of *F. hepatica* comprises of a single continuous layer of epithelial cells which show considerable variation in fine structure (34). The cell can be classified into three groups. group-I, II and III. The group-I cells are considered to be predominantly secretory in function, they contain numerous secretory granules at the apical surface, RER appears to be numerous and long. The group-II cells have absorptive function, they contain a small number of secretory granules, but have numerous cytoplasmic bodies, RER and mitochondria. The group-III cells are found predominantly lining the main caeca, their function may be more concerned with the movement of material back and forth within the lumen of the digestive tract, but they can perform both secretory and absorptive function at the same time. It is evident from this study that the histological features of type-1 epithelial cell are described herein is similar to that of type-I cell of *F. gigantica* described by Meepool (1994), and to group-I cell of *F. hepatica* described by Robinson and Threadgold (1975). The cytoplasm of type-1 epithelial cell is densely stained, contains numerous dense granules, and shows many stereocilia. In contrast, the cytoplasm of the type-3 epithelial cell shows paler cytoplasm than that of type-1 cell which is also vacuolated and has dense granules

scattered throughout the cytoplasm. These features are similar to those of type-II cell of *F. gigantica*, and group-II cell of *F. hepatica*. In our study, type-2 epithelial cell has moderately stained cytoplasm and appears paler than the type-1 cell. The number of secretory granules is lower than that of type-1 cell, but is higher than type-3 cell. In earlier study, Meepool (1994) did not find the type-2 cell, probably because of the using different fixatives, the thickness of the sections and the staining method (33). Our study reveals that the distended part of the caeca is found mainly on the main caecum of the adult stage and all parts of the caeca of the juvenile parasite where the epithelium comprise mostly type-3 of cells. It is possible that group-III cells of *F. hepatica* are comparable to the cells in the main caecum of *F. gigantica*.

In addition, Meepool (1994) concluded that the caecal epithelium cell of *F. gigantica* has only one cell type that transform into the absorptive and secretory phase alternatively (33). The cells in the absorptive phase are comparable to type-I cell and the cell in the secretory phase is type-II cell. In contrast, the present study shows only the secretory phase that occurs cyclically. It is difficult to determine the nature of the absorptive phase by light microscopy, thus, further study has to be performed by using biochemical technique and electron microscopy. However, it is apparent from this study that all 3 types of cells have many stereocilia which indicate that all types of cells may need to increase the surface area for absorptive function.

In metacercarial stage, the epithelial cells are quite different from those of the adult and juvenile. These cells are completely filled with many large, oval and very dense granules in comparison to those of adult and 50-day-old *F. gigantica*. The euchromatic nuclei are located in the basal part of the cell and show a prominent nucleolus Bennett (1975) reported that the cytoplasm of epithelial cell of NEJ

possessed numerous large (1.3 μm), dense secretory granules produced and stored during the metacercarial stage (46). The number of large secretory granule was reduced after post-excystment when the parasites start to penetrate through the gut wall, and migrate in the abdominal cavity. After the juvenile parasites penetrated the liver capsule, the caecal cells were transformed into an adult-like morphology, where the large (1.3 μm) granules were lost and Golgi complex produced a smaller (0.5μm) secretory granules (46). Early histochemical studies revealed that proteinase activity was associated with cells of the caecal epithelium (70), and it was concluded that proteinases were synthesized and packaged in the secretory granules within the caecal epithelial cells (65, 71). It is possible that the large and small granules, which are present in different stages of parasite, may contain different enzymes. Basing on previous reports, cathepsin B is predominant proteinase released by NEJ, while cathepsin L is the main secretory enzyme of the adult stage (30, 68, 86). Hence, it is possible that the large granules found in metacercarial stage contain enzyme Cat B, which are released and used by NEJ parasites to digest host materials during penetration and migration phase. Once the parasites reach the bile duct, new caecal epithelial cells may be developed and switch on the production of Cat L. Alternatively, the caecal epithelial cells may not change from the original population but only their genes are changed from Cat B to Cat L expression. This point still remained to be studied further.

2. Expression of Cathepsin L Gene in the Digestive Tract of *Fasciola gigantica* as Studied by *In Situ* Hybridization

In situ hybridization has emerged as a valuable tool for the identification of individual cells expressing specific genes (87). Recently, this method has become sufficiently sensitive to detect mRNAs present at the level of only a few molecules per cell. When mRNAs are expressed in only a small fraction of the cells in a mixed population, *in situ* hybridization may be the most sensitive nucleic acid hybridization technique available to detect a specific gene expression, such as Cat L gene. In the present study, the localization of Cat L gene expression in the digestive tract at each developmental stages of *F. gigantica* was examined Digoxigenin-labeled cDNA probe was produced by random priming method for the detection of mRNA coding for *F. gigantica* Cat L. *In situ* hybridization studies have demonstrated that the Cat L mRNA was present in abundance in the cytoplasm of the caecal epithelium in 50-day-old juvenile and adult stage. In contrast, epithelium covering the remaining parts of digestive tract, including oral sucker, buccal tube, pharynx, esophagus and caecal bifurcation, which are tegumental-type epithelium, did not exhibit the presence of Cat L mRNA This suggests that Cat L mRNAs are expressed specifically in the cytoplasm of the caecal epithelial cell of the juvenile and adult stage, which are the digestive-type epithelium. It is believed that this mRNAs carry the information from Cat L gene to the ribosomes, where the corresponding enzyme Cat L can be synthesized, and packaged in the cytoplasm of the caecal epithelial cell. And upon being stimulated by the presence of food, the enzyme is released into the caecal lumen for nutrient digestion.

In addition to the present study, our group (Grams. *et al.*, 2000) has also revealed by molecular analysis and DNA sequence of gene encoding members from the cathepsin L in *F. gigantica* that there may be at least five members of Cat L in the family. All these allelic Cat L gene have been shown by RNA *in situ* hybridization to be expressed in the epithelial gut cell of adult *F. gigantica* (88).

Unlike in the adult and juvenile stages. Cat L mRNA could not be detected in the digestive epithelium of the metacercariae. This suggests that Cat L genes are not expressed in the metacercarial stage. In agreement with the histological observations on the development of the caecal epithelial cells. Cat L genes may only start to express when the digestive system become functional after the parasites reach the liver. To prove this point further, similar *in situ* hybridization experiment should be performed by using cDNA probe for mRNA Cat B.

3. Localization of Cathepsin L in the Digestive Tract of *Fasciola gigantica*

Previously, immunolocalization of enzyme cathepsin L (Cat L) of the trematode parasite has been studied. Monoclonal antibodies against the *Schistosoma mansoni* Cat L were used to localize the enzyme in the gastrodermis of the adult parasite (24). In *Fasciola hepatica*, localization studies have demonstrated that Cat L proteinases are expressed in the caecum and a major component of excretory/secretory products (89, 90). Using immunocytochemical method, a cysteine proteinase with properties similar to Cat L-like enzymes have been localized in secretory vesicles

within the caecal epithelial cells and the host blood cells in the caecal lumen of *F. hepatica*, thus supporting a role in digestion of these enzymes (65, 66).

In the present study, the presence of Cat L enzyme which is the product of Cat L gene localized earlier was detected in the digestive epithelium by indirect immunofluorescence studies, using mouse monoclonal antibody, and polyclonal mouse anti-*F. gigantica* Cat L and sheep anti-*F. hepatica* Cat L as probes. From our observations, these antibodies give similar results. The enzyme Cat L was localized in the caecal epithelial cells in both 50-day-old juvenile and adult stages. The cytoplasm of epithelial cells showed bright fluorescence that appeared as abundant finely granular materials in the apical cytoplasm, and large intensely fluorescent globules in the caecal lumen. Our observations suggest that Cat L is expressed specifically in the caecal epithelial cells in juvenile and adult stages. After being released into the lumen of the caecum, the enzyme is mixed with caecal contents for nutrient digestion; this explains why strong fluorescence was also observed in the lumen.

In the immunolocalization experiments using polyclonal antibodies against *F. hepatica* Cat L and *F. gigantica* Cat L, similar results were observed. Thus, it is likely that *F. gigantica* Cat L is the most likely homologues of the *F. hepatica* Cat L. Recently, Spithill *et al.*, (1997) have reported 94.2% homology between Cat L cDNA from a *F. gigantica and* Cat L cDNA of *F. hepatica* (FhCat L) (90).

In addition to the presence the caecal epithelium, Cat L has been localized in the reproductive organs of *S. mansoni*. Michel and colleague (1995) suggested that Cat L role might also be associated with the cross-linking of eggshell proteins (24). Furthermore, Spithill and Morrison (1997) also localized Cat L in the Mehlis' gland of *F. hepatica* as well as in the caecum. These findings suggest that apart from playing a

role in feeding (66). Cat L proteinase may also be involved in egg production, possibly through the processing of eggshell precursor proteins. In the present study, Cat L could not be localized in Mehlis' gland and other reproductive organ of *F. gigantica*. It is possible that the different in the result could be due to different isotypes of Cat L that may be present in the Mehlis' gland of *F. gigantica*. Several researchers have screened cDNA libraries and isolated full-length encoding gene for adult *Fasciola* cysteine proteinase (86, 55), and proven that the secreted adult proteinases are of the cathepsin L class. Heussler and Dobbelaere (1994) could isolated cDNA clones and gene fragments encoding five different cathepsin L proteinases that have homology ranging from 48 to 84%, and thus demonstrated the presence of a multigenic family of Cat L in *F. hepatica* (61). In *F. gigantica* at least five similar multigenic family of Cat L was also demonstrated by Grams *et al.*, (2000) (88).

In the present study, the metacercarial stage did not exhibit specific fluorescence staining for Cat L in any tissue after staining by both types of antibodies. This suggests that metacercaria and NEJ may synthesize different cathepsin enzymes Wilson, et al. (1998) has shown that in *F. hepatica* Cat B is the predominant cathepsin proteinase released by NEJ, while Cat L is a major secretory enzyme of the adult fluke (65, 86, 68). Cat B was detected in the parenchymal tissue of the adult fluke, and it was localized to the gut epithelial cells of NEJ (72). In NEJ, the principal function of secreted Cat B may be to assist the parasite in invasion and migration. The NEJ Cat B may not function as a digestive enzyme since the juvenile fluke caecum may possess only secretory function, while the absorptive function may arise later in late juvenile and the adult stages (83). Like NEJ, the caecal epithelial cells of metacercarial stage may synthesize only Cat B. From the data present in this study, it is possible to

conclude that Cat L is expressed only in late juvenile and adult stages, which may be associated with changes in environment and diet during the migration of the fluke, while in the metacercaria and NEJ another group of proteinase, such as Cat B could be expressed for the specific purpose of penetration and migration in the host tissue. In addition, it can be concluded that the differential production of enzymes can be linked with the morphological changes in the caecal epithelium during various stages of development.

CHAPTER VII

CONCLUSIONS

1. Structure and histology of the digestive tract of *Fasciola gigantica*

1.1 The digestive tracts of adult and 50-day-old *F. gigantica* contains two types of epithelium: the tegumental-type and digestive-type.

1.2 The proximal part of the digestive tract, including oral sucker, buccal tube, pharynx, esophagus and part of the tract anterior to caecal bifurcation are lined by tegumental-type epithelium, whereas the caecum is lined by the digestive-type epithelium.

1.3 The tegumental-type epithelium is a syncytium distinguishable by numerous basal infoldings extending towards the apical surface that is corrugated with cross sections of ridges.

1.4 The digestive-type epithelium is composed of columnar or cuboidal cells with round and basally-located euchromatic nuclei. The cells have prominent nucleoli, and their apical surfaces possess numerous stereocilia.

1.5 The digestive-type epithelial cells could be classified into 3 types according their staining properties, abundance of zymogen granules, and height: type-1, type-2 and type-3 cells, whose cytoplasm are densely, moderately and lightly stained by basic

dye consecutively. The number of secretory granules and height of the cells are decreased in the same order. All 3 type of cells are found in the same area, but the number of each type depends on the amount of food present in the caecal lumen.

1.6 Type-1 cell is in the synthetic phase that produces and stores a lot of zymogen granules in the cytoplasm when a small amount of food is present in the caecal lumen. Type-2 and 3 cells are in secretory phase, in which the secretory granules are exocytosed and the enzyme is released for digestion of a larger amount of food present in the caecum. It is likely that all 3 types of cells may belong to one group, but function in different phases, and that all of them can perform both the digestive and absorptive functions.

1.7 The caecal epithelial cells of metacercariae are distinct from those of the adult and juveniles worms. These cells are completely filled with large and very dense granules when compared to those observed in the epithelial cells of adult and 50 day-old juveniles. Each cross section of the caecal lumen appears very irregular in shape, and is surrounded by only a few epithelial cells.

2. Expression of cathepsin L gene in the digestive tract of *Fasciola gigantica* as studied by *in situ* hybridization

2.1 Cathepsin L mRNA is expressed specifically in the caecal epithelial cells in adult and 50 day-old juvenile stages of *F. gigantica*, and are located in the digestive-type epithelium, whereas the tegumental-type epithelium which line the proximal part of the digestive tract and tissues outside the digestive system is not labeled.

2.2 Cathepsin L mRNA could not be detected in the metacercarial digestive epithelium, hence this enzyme was thought to be expressed only in late juvenile and adult parasites.

3. Localization of cathepsin L in the digestive tract of *Fasciola gigantica*

3.1 When using mouse monoclonal antibody to Cat L and polyclonal mouse anti-Cat L *F. gigantica* and sheep anti-Cat L *F. hepatica* as primary antibody probe to localize Cat L, the results appear similar. The enzyme Cat L is specifically localized in the cytoplasm of the caecal epithelium cells and caecal lumen of 50-day-old and adult flukes.

3.2 Metacercariae of *F. gigantica* do not exhibit the presence of enzyme Cat L in any tissue after staining with both types of antibodies. Hence, this immunofluorescence results support the in situ hybridization experiment and confirm the age and tissue specificity of Cat L gene expression, and the production of the enzyme itself.

REFERENCES

1.Over HJ. Ecological basis of parasite control: trematodes with special reference to fascioliasis Vet Parasitol 1982; 2: 85-97.

2.Boray JC Flukes of domestic animals. In: Gaafar SM, Howard WE, Marsh RE, editors Parasites, Pests and Predators. New York: Elsevier; 1985. 179-218.

3.Edney JM, Muchlis A. Fascioliasis in Indonesian live stock. Commun Vet 1962; 6: 49-52.

4.Torgerson P, Claxton J. Epidemiology and control. In: Dalton JP, editor. Fasciolosis. UK: Cambridge University Press; 1999. 113-39.

5.Soesetya RHB The prevalence of *Fasciola gigantica* infection in cattle in East Java, Indonesia. Malays Vet J 1975; 6: 5-8.

6.Fabiyi JP. Production losses and control of helminths in ruminants of tropical region. Int J Parasitol 1987; 17: 435-42

7.Pholpark M, Srikitjakarn L. The control of parasitism in swamp buffalo and cattle in Nort-East Thailand. In: International Seminar on Animal Health and Production Services for Village Livestock. Knon Kaen, Thailand; 1989. p.244-9

8.Anon. Control of Foodborn, Trematode Infections. Geneva: WHO; 1995. WHO Technical Series. No. 849. p.157.

9.Srihakim S, Pholpark M. Problem of fasciolosis in animal husbandry in Thailand. Southeast Asian J Trop Med Pub Hlth 1991; Suppl 22. 352-5.

10.Kachintorn U, Atisook K, Tesjaroen S, Lertakyamanee N, Plengvanit U. *Fasciola gigantica*. the second case of human infection in Thailand. J Med Assoc Thai 1988; 71: 451-5.

11.Kyrönseppa HJ, Goldsmid JM. Studies on the intestinal parasites in African patients in Owamboland, South West Africa. Trans R Soc Trop Med Hyg 1978; 72: 16-21.

12.Boray JC, Crowfoot PD, Strong MB, Allison JR, Schellenbaum M, von Orelli M, Sarasin G. Treatment of immature and mature *Fasciola hepatica* infections in sheep with triclabendazole. Vet Rec 1983, 113: 315-7.

13.Overend DJ, Bowen FL. Resistance of *Fasciola hepatica* to triclabendazole. Aust Vet J 1995; 72: 275-6.

14.Haroun ET, Hillyer GV Resistance to fascioliasis. A review. Vet Parasitol 1986; 20: 63-93.

15.Yoshihara S, Nakagawa M, Suda H. Protection against *Fasciola gigantica* infection in rats administered metacercarial antigens. Res Vet Sci 1985; 39: 383-4.

16.Estuningsih SE, Smooker PM, Wiedosati E, Widjajanti S, Vaiano S, Partoutomo S, Spithill TW. Evaluation of antigens of *Fasciola gigantica* as vaccines against tropical fasciolosis in cattle. Int J Parasitol 1997; 27: 1419-28.

17.Kirschke H, Langner J, Riemann S, Wiederanders B, Ansorge S, Bohley P. Lysosomal cysteine proteinases. In. Protein degradation in health and disease. Ciba Found Symp 1979, 75: 15-35.

18.Katunuma N, Kominami E. Structure and functions of lysosomal thiol proteinases and their endogenous inhibitors. Curr Top Cell Regul 1983; 22: 71-101.

19.Kominami E, Tsukahara T, Bando Y, Katunuma N. Distribution of cathepsins B and H in rat tissues and peripheral blood cells. J Biochem 1985; 98: 87-93.

20.Bando Y, Kominami E, Katunuma N. Purification and tissue distribution of rat cathepsin L. J Biochem 1986; 100: 35-42.

21.Maki J, Yanagisawa T. Demonstration of carboxyl and thiol protease activities in adult *Schistosoma mansoni*, *Dirofilaria immitis*, *Angiostrongylus cantonensis*, and *Ascaris suum*. J Helminthol 1986; 60: 131-7.

22.Zussman RA, Bauman PM, Petruska JC. The role of ingested hemoglobin in the nutrition of *Schistosoma mansoni*. J Parasitol 1970; 56: 75-9.

23 McKerrow JH, Doenhoff MJ. Schistosome proteases. Parasitol Today 1988; 4: 334-40.

24.Michel A, Ghoneim H, Resto M, Klinkert MQ, Kunz W. Sequence, characterization and localization of a cysteine proteinase cathepsin L in *Schistosoma mansoni*. Mol Biochem Parasitol 1995; 73: 7-18.

25.Chapman CB, Mitchell GF. Proteolytic cleavage of immunoglobulin by enzymes released by *Fasciola hepatica*. Vet Parasitol 1982; 11: 165-178.

26.McKerrow JH, Newport G, Fishelson Z. Recent insights into the structure and function of a larval proteinase involved in host infection by a multicellular parasite. Pro Soc Exp Biol Med 1991; 197: 119-124.

27.Howard RJ, Chapman CB, Mitchell GF. A difference in surface proteins of *Fasciola hepatica* lavae from intact and nude mice. Aust J Exp Biol Med Sci 1980; 58:201-5.

28.Wijffels GL, Salvatore L, Dosen M, Waddington J, Wilson L, Thompson C, Campbell N, Sexton J, Wickel J, Bowen F, Friedel T, Spithill TW.

Vaccination of sheep with purified cysteine proteases of *Fasciola hepatica* decreases worm fecundity. Exp Parasitol 1994; 78: 132-48.

29.Dalton JP, McGonigle S, Rolph TP, Andrews SJ. Induction of protective immunity in cattle against infection with *Fasciola hepatica* by vaccination with cathepsin L proteinases and hemoglobin. Infect Immun 1996; 64: 5066-70.

30.Smith AM, Dowd AJ, McGonigle S, Keegan PS, Brennan G, Trudgett A, Dalton JP. Purification of a cathepsin L-like proteinase secreted by adult *Fasciola hepatica*. Mol Biochem Parasitol 1993; 62: 1-8.

31.Chapell CL, Dresden HH. *Schistosoma mansoni* adult worm proteases: Tissue localization in the parasite and antibody response in infected animal. In: Molecular Strategies of Parasites Invasion. New York: A.R. Liss; 1987. p. 399-408.

32.Ray G, McKerrow JH. Gut-specific and developmental expression of a *Caenorhabditis elegan* cysteine protease gene. Mol Biochem Parasitol 1992; 51: 239-49.

33.Meepool A. Structure and histology of digestive, excretory and reproductive systems in *Fasciola gigantica*. [M.S. Thesis in Anatomy]. Bangkok: Faculty of Graduated Studies, Mahidol University; 1994.

34.Robinson G, Threadgold LT. Electron microscope studies of *Fasciola hepatica*. XII. The fine structure of the gastrodermis. Exp Parasitol 1975; 37: 20-36.

35.Nobel ER, Nobel GA, Schad GA, MacInnes AJ. Parasitology: The biology of animal parasite 6th ed. London: Philadelphia; 1989.

36.Gwadz RW, Hotez PJ. Parasitic Diseases. 3 rd ed. New York: Spinger-Verlag; 1995.

37.Dixon KE, Mwrcer EH. The fine structure of the cyst wall of the metacercaria of *F. hepatica*. Q J Microsc Sci 1964; 105:385-9.

38.Dixon KE. The physiology of excystment of the metacercaria of *Fasciola hepatica* L. Parasitology 1966; 56 431-56.

39.Andrews ST. The life cycle of *Fasciola hepatica*. In: Dalton JP, editor. Fasciolosis. UK: Cambridge University Press; 1999. 1-30.

40.Smyth JD. Introduction of animal parasitology. 2nd ed. New York: John Wiley&Son; 1967.

41.Malex EA. Snail-transmitted parasite diseases. Vol II. Florida: CRC Press; 1992.

42.Rees G. Light and electron microscope studies of the redia of *Parorchis acanthus* Nicoli. Parasitology 1966; 56: 589-602.

43.Wilson RA. Gland cells in the redia of *Fasciola hepatica*. Parasitology 1972; 65: 433-6.

44.Eramus DA. The host-parasite interface of trematodes. In. Dawes B, editor. Advance in parasitology. New York: Academic Press; 1977 201-43.

45.Dawes B. On the growth and maturation of *Fasciola hepatica* L in the mouse. J Helminthol 1962; 36: 11-38.

46.Bennett CE. *Fasciola hepatica*: development of caecal epithelium during migration in the mouse. Exp Parasito 1975; 37: 426-41.

47.Dawes B. A histological study of the caecal epithelium of *Fasciola hepatica* L. Parasitology 1962; 52: 483-93.

48.Spence IM, Silk MH. Ultrastructural studies of the blood fluke-*Schistosoma mansoni*. IV. The digestive System. S Afr J Med Sci 1970; 35: 93-112.

49.Shannon WA Jr, Bogitsh BJ. Cytochemical and biochemical observations on the digestive tracts of digenetic trematodes. V. Ultrastructure of *Schistosomatium douthitt* gut. Exp Parasitol 1969; 26: 344-53.

50.Bogitsh BJ. Cytochemical and biochemical observations on the digestive tracts of digenetic trematodes. IX. *Megalodiscus temperatus*. Exp Parasitol 1972; 32: 244-60.

51.McKerrow JH. Parasite proteases. Exp Parasitol 1981; 68: 111-5.

52.Tort J, Brindely PJ, Knox D, Wolfe KH, Dalton JP Proteinases and associated genes of parasitic helminths. Adv Parasitol 1999; 43: 161-266.

53.Spithill TW, Dalton JP. Progress in development of liver fluke vaccines. Parasitol Today 1988; 14: 224-8.

54.Rege AA, Herrera PR, Lopez M, Dresden MH. Isolation and characterization of a cysteine proteinase from *Fasciola hepatica* adult worms. Mol Biochem Parasitol 1989; 35: 89-95.

55.Yamasaki H, Aoki T, Oya H. A cysteine proteinase from the liver fluke Fasciola spp: purification, characterization, localization and application to immunodiagnosis. Jpn J Parasitol 1989; 38: 373-84.

56.Dowd AJ, Smith AM, McGonigle S, Dalton JP. Purification and characterization of a second cathepsin L proteinase secreted by the parasitic trematode *Fasciola hepatica*. Eur J Biochem 1994; 223: 91-8.

57.Spithill TW, Smooker PM, Copeman DB. *Fasciola gigantica*: epidermiology, control, immunology and molecular biology. In: Dalton JP, editor. Fasciolosis. UK: Cambridge University Press; 1999. 465-509.

58.Fagbemi BO, Hillyer GV. Partial purification and characterization of the proteolytic enzymes of *Fasciola gigantica* adult worms. Vet Parasitol991; 40: 217-26.

59.Fagbemi BO, Hillyer GV. The purification and characterization of a cysteine protease of *Fasciola gigantica* adult worms. Vet Parasitol 1992, 43: 223-32.

60.Panaccio M, Trudgett A. Molecular biology. In: Dalton JP, editor. Fasciolosis. UK: Cambridge University Press; 1999. 449-64

61.Heussler VT, Dobbelaere DA. Cloning of a protease gene family of *Fasciola hepatica* by the polymerase chain reaction. Mol Biochem Parasitol 1994, 64: 11-23

62.Roche L, Dowd AJ, Tort J, McGonigle S, UcSweeney A, Curley P, Ryan T, Dalton JP. Functional expression of Fasciola hepatica cathepsin L1 in Saccharomyces cerevisiae. Eur J Biochem 1997; 245: 373-80.

63.McGinty A, Moore M, Halton DW, Walker B. Characterization of the cysteine proteinases of the common liver fluke *Fasciola hepatica* using novel active-site directed affinity labels. Parasitology 1993; 106: 487-93.

64.Berasain P, Goni F, McGonigle S, Dowd A, Dalton JP, Frangione B, Carmond C. Proteinases secreted by *Fasciola hepatica* degrade extracellular matrix and basement membrane components. J Parasitol 1997; 83: 1-5.

65.Smith AM, Dowd AJ, Heffernan M, Robertson CD, Dalton JP. *Fasciola hepatica*: a secreted cathepsin L-like proteinase cleaves host immunoglobulin. Int J Parasitol 1993; 23: 977-83.

66.Spithill TW, Morrison CA. Molecular vaccines for control of *Fasciola hepatica* infection in ruminants. In: Boray J, editor. Immunology Pathophysiology and control of fasciolosis. Newjersy: Merk AGVET, Rashway. 1997.

67.Carlos C, Dowd AJ, Smith AM, Dalton JP. Cathepsin L proteinase secreted by *Fasciola hepatica in vitro* prevents antibody-mediated eosinophil attachment to newly excysted juveniles. Mol Biochem Parasitol 1993, 62: 9-17.

68.Wilson LR, Good RT, Panaccio M, Wijffels GL, Sandeman RU, Spithill TW. *Fasciola hepatica*: Characterization and cloning of the major cathepsin B protease secreted by newly excysted juvenile Liver fluke. Exp Parasitol 1998; 88: 85-94.

69.Chung YB, Kong Y, Joo IJ, Cho SY, Kang SY. Excystment of *Paragonimus westermanti* metacercariae by endogenous cysteine protease J of Parasitol 1995; 81: 137-42.

70.Howell MJ. Localization of proteolytic activity in *Fasciola hepatica*. Journal of Parasitology 1973; 59: 454-6.

71.Yamasaki H, Kominami E, Aoki T. Immunocytochemical localization of a cysteine protease in adult worms of the liver fluke *Fasciola* sp. Parasitol Res 1992; 78. 574-80.

72.Creaney J, Wilson L, Dosen M, Sandeman M, Spithill TW, Parsons JC. *Fasciola hepatica*: irradiation induced alterations in carbohydrate and cathepsin B protease expression in newly excysted juvenile liver fluke. Exp Parasitol 1996; 83. 202-15.

73.Viyanant V, Upatham ES, Sobhon P, Krailas D, Ardseungnoen P, Anantawara S. Development and characterization of monoclonal antibodies against

excretory-secretory antigens of *Fasciola gigantica*. Southeast Asian J Trop Med Public Health 1997; 28 (Suppl 1). 128-33.

74.Kiatpathomchi K. Purification and characterization of liver-fluke *Fasciola gigantica* antigens [M.S. Thesis in Biochemistry] Bangkok: Faculty of Graduate Studies, Mahidol University. 1995.

75.Guralnick G, editor. Stratagene cloning system catalog. United Kingdom; 1999.

76.Maniatis T, Fritsch EF, Sambrook J. Molecular cloning (a laboratory manual). New York: Cold Spring Harbor Laboratory; 1982.

77.Peacock AC, Dingman CW. Molecular weight estimation and separation of ribonucleic acid by electrophoresis in agarose-acrylamide composite gels. Biochemistry 1968; 7: 668-74.

78.Feinberg AP, Vogelstein B. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 1983; 132: 6-13.

79.Feinberg AP, Vogelstein B. Feinberg B. Addendum: A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 1984; 137. 266-267.

80.Watanabe S. A revision of genus *Fasciola* in Japan, with particular reference to *F. hepatica* and *F. gigantica*. In: Kacru M, Yoshitoka K, Hisokichi M, editors. Progress of medical parasitology in Japan vol II. Tokyo: Megano Parasitology Museum, 1965. 361-81.

81.Halton DW. Observations on the nutrition of digenetic trematodes. Parasitol 1967; 57. 639-60

82.Kumkate S. Development of *Fasciola Gigantica* (Cobbold, 1856) in mice and characterization of a monoclonal antibody against adult worm antigen. [M.S. Thesis in Environment Biology]. Bangkok: Faculty of Graduate studies, Mahidol University; 1999.

83.Bennett CE, Threadgold LT. Electron microscope studies of *Fasciola hepatica*. XIII. Fine structure of newly excysted juvenile. Exp Parasitol 1973; 34: 85-99.

84.Sobhon P, Upatham ES. Snail hosts, lifecycle, and tegumental structure of oriental Schistosomes. Bangkok: Lincoln Promotion: 1990.

85.Sobhon P, Anantavara S, Dangprasert T, Viyanant V, Krailas D, Upatham ES, Wanichanon C, Kusamran T. *Fasciola gigantica*: studies of the tegument as a basis for the development of immunodiagnosis and vaccine. Southeast Asian J Trop Med Public Health 1998; 29 (2): 387-400.

86.Wijffels GL, Panaccio M, Salvatore L, Wilson L, Walker ID, Spithill TW. The secreted cathepsin L-like proteinases of the trematode, *Fasciola hepatica*, contain 3-hydroxyproline residues. Biochem J 1994; 299: 781-90.

87.Angerer LM, Cox KH, Angerer RC. Demonstration of tissue-specific gene expression by *in situ* hybridrization. Methods Enzymol 1987; 152: 661-72.

88 Grams R, Grams SV, Upatham ES, Sobhon P, Viyanant V. Cloning and characterization of antigen-encoding genes from *Fasciola gigantica*. In: Proseeding of the 2nd FIMSA congress, Immunology and Allergology in the Asia-Oceania: at the dawn of the new millenium; 2000 Jan 23-27; Bangkok, Thailand: Aksornsmai Press; 1999. p.182.

89. Yamasaki H, Aoki T. Cloning and sequence analysis of the major cysteine protease expressed in the trematode parasite Fasciola sp. Biochem Mol Biol Int 1993; 31: 537-42.

90. Spithill TW, Piedrafita D, Smooker PM. Immunological approaches for the control of fasciolosis. Int J Parasitol 1997; 27:1221-35.

APPENDIX

Appendix 1: Fixatives

1.1 Alcohol-Formal-Acetic (AFA) Fixative

Formalin (40% formaldehyde)	10	part
95% ethyl alcohol	50	part
Acetic acid, glacial	2	part
Distilled water	40	part

1.2 Bouin's Fluid

Picric acid, sat.aq. solution	75	part
Formalin (40% formaldehyde)	25	part
Acetic acid, glacial	5	part

1.3 Kanovky's Fixative

4% glutaraldehyde solution

2% paraformaldehyde

0.1 M phosphate buffer saline (Mallonig, 1961)

1.3 1% Osmium Tetroxide (O_3SO_4) in 0.1 M PBS (Mallonig, 1961)

1.4 4% Paraformaldehyde in 0.1 M PBS for Indirect Immunofluorescence and *In Situ* Hybridization

Appendix 2: Dyes

2.1 Semichon's Carmine (Stock Solution)

Dissolved carmine in 50% glacial acetic acid in distilled water. Until the carmine crystal not dissolve then heat at 95-100°C for 15 minutes and filter. Working solution is 1 part of stock solution and 1 part of 70% ethyl alcohol.

2.2 Hematoxylin & Eosin

Harris's Alum Hematoxylin

Hematoxylin crystals	5	g.
95% ethyl alcohol	50	ml.
Potassium alum	100	g.
Distilled water	1000	ml.
Mercuric oxide	2.5	g.

Dissolve potassium alum in water by the aid of stirring and heat, remove and mix with the solution of hematoxylin in ethyl alcohol. Add the mercuric oxide slowly during the mixture is still hot. Until it becomes dark purple, the container is quickly moved to cool in the water.

1% Stock Alcoholic Eosin

Eosin Y, water soluble	1	g.
Distilled water	20	ml.
Ethyl alcohol	80	ml.

Dissolve Eosin Y in distilled water and then add ethyl alcohol. Working solution is 1 part of stock Eosin solution and 1 part of 95% ethyl alcohol. Before use added 0.5 ml glacial acetic acid per 100 ml of working solution.

2.3 1% Methylene Blue in 1% Borax in Distilled Water

Methylene blue	2	g
Distilled water	200	ml
Sodium tetraborate ($Na_2B_4O_7$ $10H_2O$)	3 8	g

Appendix 3: Embedding Media

3.1 Paraplast (melting point 56 °C) for Paraffin Embedding

3.2 Araldite 502 for Conventional Embedding

Araldite 502	27	ml
DDSA	20	ml
Accelerator DMP-30	1	ml

Appendix 4: Reagents

4.1 0.2 M Phosphate Buffer Saline (PBS) (Mallonig, 1961), pH 7.4

Solution A : Monobasic sodium phosphate ($NaH_2PO_4.H_2O$)

4.52 g. in 100 ml H_2O

Solution B . Sodium hydroxide (NaOH)

5.04 g. In 100 ml H_2O

Mix solution A · B = 41 5 ml : 8.5 ml. Adjust to final volume = 65 ml and add 1.105 g sodium chloride (NaCl).

4.2 Reagents for Isolation of Plasmid DNA from *E. coli* (Small-Scale Preparations of Plasmid DNA by the Alkaline Lysis Method)

LB Medium

Bacto tryptone 10 g

Bacto tryptone 5 g

NaCl 5 g

Solution I

25 mM Tris-HCl, pH 8

50 mM glucose

10 mM EDTA

Solution II (freshly prepare)

0.1 N NaOH

1% SDS

Solution III

2 7 M potassium acetate, pH 4.8

TE Buffer

10 mM Tris-HCl, pH 8

1 mM EDTA

4.3 Reagents for Agarose Gel Electrophoresis

Tris-borate-EDTA Running Buffer (5X)

Tris base 52 g

Boric acid 27.5 g

Disodium EDTA.$2H_2O$ 4.65 g

Water to 1 liter (Adjust pH 8 3)

Sample Loading Solution (10X)

50% Glycerol

0.25% Bromophenolblue

0.25% Xylene cyanole FF.

Working solution (0.5X): add Ethidium bromide to 0.5 μg/ml final concentration

4.4 Reagents for *In Situ* Hybridization

DEPC-Treated Water

Add DEPC to a final concentration of 0.1% to distilled water, mix well, leave overnight, autoclave

DEPC-Treated PBT

DEPC-treated PBS

0.2% Tween 20

DEPC-Treated PBS

140 mM NaCl

2 7 mM KCl

10 mM Na_2HPO_4

1.8 mM KH_2PO_4

dissolved in DEPC-treated-H_2O

Prehybridization Buffer

4XSSC

2% blocking solution (blocking stock solution 1:5, DIG labeling and detection kit)

0.02% SDS

50% deionized formamide

200 µg/ml denatured and sheared salmon sperm DNA

Hybridization Buffer

4xSSC

10% dextran sulfate

1% blocking solution (blocking stock solution 1:10, DIG labeling and detection kit)

0.02% SDS

50% deionized formamide

200 µg/ml denatured and sheared salmon sperm DNA

SSC (Sodium Chloride-Sodium Citrate)

150 mM NaCl

15 mM Na-citrate pH 7.2

Washing Buffer

100 mM Tris-HCl pH 7.5

150 mM NaCl

Blocking Solution

Washing buffer

0.1% Tween 20

2% normal sheep serum

Detection Buffer

100 mM Tris-HCl pH 7.9, 10 mM NaCl, 50 mM $MgCl_2$.

4.5 Reagent for Preparation of Microscope Slides and Siliconized Cover Slips

Chromic Acid Solution	**Silicone Solution**
10% potassium dichromate ($K_2Cr_2O_7$)	2 ml siliconizing/silicon stock
4% conc. H_2SO_4	300 ml distilled water

Appendix 5 : Preparation of Microscope Slides and Cover Slips

5.1 Preparation of Gelatin-Chromalum Coated Microscope Slides for *In Situ* Hybridization and Indirect Immunofluorescence

Glass slides were cleaned by soaking in 95% ethanol for 1 hour. Gelatin solution was prepared by the following procedure. 10 g Gelatin was dissolved in 1 liter of distilled water and heated at 40-50°C. Then 25% chromium potassium sulfate (CPS) solution was added to the gelatin to making a final concentration of 0.1% CPS. The slides were dipped in gelatin solution for 10 minutes, then let the slides air dried and baked at 60°C overnight. Finally, the gelatin-coated slides were stored under dry and dust-free conditions.

5.2 Preparation of Siliconized Cover Slips

Coverslips were immersed into chromic acid solution for 1 hour. Then the coverslip were washed in distilled water for 5 minutes before placing them into silicone solution for about 2-3 minutes and dried in a hot air oven for 30 minutes.

Appendix 6 :. DNA sequences of *Fasciola gigantica* cathepsin L(Cat L1)

(http://www.ncbi.nlm.nih.gov)

1 atgcgattgttcatattagccgtcctcacggtcggagtgcttggctcgaatgatgatttgtggcatcaatggaagcgaatgtac

85 aataaagaatacaatggggctgacgatgagcacagacgaaatatttgggaagagaatgtgaaacatatccaagaacataa

165 cctacgtcacgatctcggcctcgtcacctacacattgggattgaaccaattcactgatatgacattcgaggaattcaaggcc

246 aaatatctaacagaaatgccacgcgcgtccgatatactctcacacggtatcccgtatgaggcgaacaatcgtgccgtacccg

328 acaaaattgactggcgtgaatctggttatgtgacggagttgaaagatcagggaaattgtggttcatgttgggcattctcaac

410 aaccggtactatggaaggacagtatatgaaaacgaaagaactagtatttcattctctgagcaacaactggtcgattgtagcggt

494 ccttggggaaatatgggttgcagtggtggattgatggaaaatgcttacgaatatttgaaacaatttggattggaaaccgaat

576 cctcttatccgtacacggctgtggaaggtcagtgtcgatacaataggcagttgggagttgccaaagtgacggactactatac

658 tgtgcattctggcagtgaggtagaattgaaaaatctagtcggtgccgaaggacctgctgcggtcgctgtggatgtggaat

738 ctgacttcatgatgtacagtggtggtatttatcaggccgaacttgtcatcgcttcgtgtgaatcatgcagtcttggctgtcggtt

827 atggaacacaggtggtactgctattggattgtgaaaaatagttggggatcgtcctggggtgagcgtggttacattcgaatgg

912 ttaggaaccgaggtaactgtgtggaattgcttcgctggccagtctcccgatggtggccacgatttccgtga

Appendix 7 : Restriction sites were generated for the cathepsin L Gene Fragment by Using a Webcutter 2.0 Programe

(http://www.firstmarket.com/cutter/cut2.html)

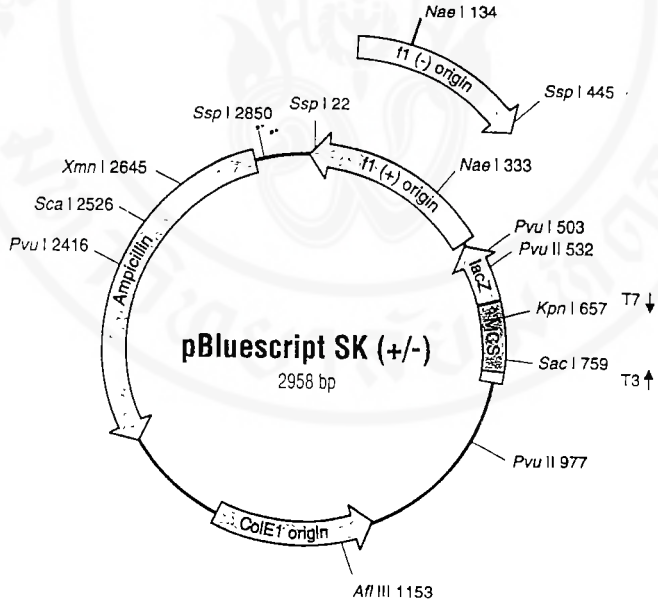
Table by Enzyme Name

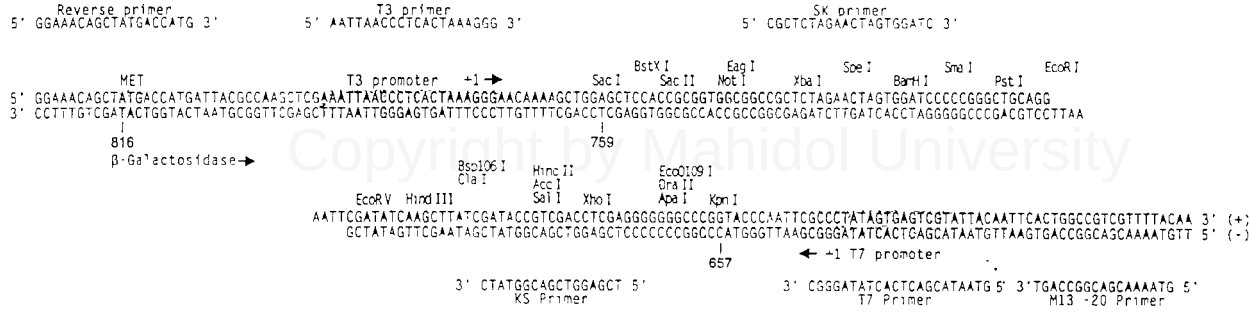
Enzyme name	No. cuts	Positions of sites	Recognition sequence
AccB1I	1	700	g/gyrcc
AclNI	1	451	a/ctagt
AcsI	1	235	r/aatty
AflIII	1	926	a/crygt
AgeI	1	412	a/ccggt
Alw21I	1	116	gwgcw/c

ApoI	1 235	r/aatty
Asp700I	3 211 783 939	gaann/nnttc
AspHI	1 116	gwgcw/c
BalI	1 949	tgg/cca
BanI	1 700	g/gyrcc
Bbv12I	1 116	gwgcw/c
BcgI	1 716	cgannnnnntgc
Bpu14I	1 903	tt/cgaa
BsaMI	2 405 668	gaatgc
BsaOI	2 32 722	cgry/cg
BsaWI	1 412	w/ccggw
Bse118I	1 412	r/ccggy
Bsh1285I	2 32 722	cgry/cg
BshNI	1 700	g/gyrcc
BsiEI	2 32 722	cgry/cg
BsiHKAI	1 116	gwgcw/c
BsmI	2 405 668	gaatgc
Bsp119I	1 903	tt/cgaa
Bsp1407I	2 80 752	t/gtaca
BspHI	1 746	t/catga
BspLU11I	1 926	a/catgt
BspMI	1 716	acctgc
BsrFI	1 412	r/ccggy
BsrGI	2 80 752	t/gtaca
BssAI	1 412	r/ccggy
BssT1I	1 496	c/cwwgg
BstBI	1 903	tt/cgaa
BstMCI	2 32 722	cgry/cg
Cfr10I	1 412	r/ccggy
CfrI	1 947	y/ggccr
Csp45I	1 903	tt/cgaa
DraII	1 709	rg/gnccy
DraIII	1 838	cacnnn/gtg
EaeI	1 947	y/ggccr
Eam1104I	1 138	ctcttc
EarI	1 138	ctcttc
Eco130I	1 496	c/cwwgg
Eco64I	1 700	g/gyrcc
EcoO109I	1 709	rg/gnccy
EcoRI	1 235	g/aattc
EcoT14I	1 496	c/cwwgg
ErhI	1 496	c/cwwgg
Ksp632I	1 138	ctcttc
LspI	1 903	tt/cgaa
MluNI	1 949	tgg/cca
MscI	1 949	tgg/cca
MslI	3 220 595 904	caynn/nnrtg

Mva1269I	2	405 668	gaatgc
NspI	1	930	rcatg/y
NspV	1	903	tt/cgaa
PinAI	1	412	a/ccggt
PpuMI	1	709	rg/gwccy
Psp5II	1	709	rg/gwccy
RcaI	1	746	t/catga
SfuI	1	903	tt/cgaa
SpeI	1	451	a/ctagt
SspBI	2	80 752	t/gtaca
SspI	2	126 547	aat/att
StyI	1	496	c/cwwgg
XcmI	1	957	ccannnnn/nnnntgg
XmnI	3	211 783 939	gaann/nnttc

Appendix 7 : A Map and Restriction Sites of SK-pBluescript





BIOGRAPHY

NAME	Mr. Sompong Sriburee
DATE OF BIRTH	27 November 1974
PLACE OF BIRTH	Lamphun, Thailand
INSTITUTIONS ATTENDED	Chaingmai University, 1993-1997: Bachelor of Science (Radiological Technology) Mahidol University, 1997-2000: Master of Science (Anatomy)
FOLLOWSHIP/ RESEARCH GRANT	University Development Commission Scholarship (UDC) and Thailand Research Fund (TRF) (Senior Research Scholar Fellowship to Prof. Prasert Sobhon)
POSITION&OFFICE	Department of Radiological Technology, Faculty of Associated Medical of Science, Chaingmai University.