รายงานวิจัยฉบับสมบูรณ์

โครงการ **การพัฒนาชุดทดสอบสำหรับตรวจวินิจฉัยโรค fasciolosis จากการติดเชื้อพยาธิใบไม้ในตับ *Fasciola gigantica* แบบ sandwich ELISA โดยใช้โมโนโคลนัลและโพลีโคลนัลแอนติบอดีต่อโปรตีน Cathepsin L**

Development of sandwich enzyme-linked immunosorbent assay using monoclonal and polyclonal antibodies against Cathepsin L (CatL) for diagnosis of fasciolosis caused by *F. gigantica*

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Panat Anuracpreeda

Abstract

Project Code : MRG5580012

Project Title : Development of sandwich enzyme-linked immunosorbent assay using monoclonal and polyclonal antibodies against Cathepsin L (CatL) for diagnosis of fasciolosis caused by *F. gigantica*

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Project Period : 2 years

The purpose of this research was to produce, characterize and purify the monoclonal antibody (MoAb) and polyclonal antibody (PoAb) specific against recombinant cathepsin L (rCatL) of *Fasciola gigantica* and use both antibodies to study the distribution of this antigen and application in immunodiagnosis of animal fasciolosis. Hybridoma secreting MoAb reactive against rCatL was obtained from fusion of rCatL-immunized spleen cells of BALB/C mouse with mouse myeloma cells. All clones of hybridoma that produce MoAb specific to rCatL, as assayed by ELISA and EITB analysis, were used for cross-reactivities studies and localization of the antigen in tissues of each developmental stage of *F. gigantica* by mean of indirect immunoperoxidase technique. Purified specific antibodies rCatL of *F. gigantica* were used to develop both sandwich ELISA and tested for CatL in sera of experimentally infected mice as well as naturally infected ruminants. Results showed that MoAbs showed stronger reaction with rCatL. In addition, these MoAbs also exhibited strong reaction with native CatL in whole body (WB) fraction, and excretory-secretory (ES) fraction of *F. gigantica*. Moreover, no cross-reaction was detected in the WB antigens from other parasites as compared to that from *F. gigantica*. Immunoperoxidase staining of frozen sections of adult parasites by using these MoAbs indicated that CatL was detected in both caecal epithelium and in the lumen of the caecum of metacercariae, NEJ, 1, 3, 5-week-old juveniles and adult *F. gigantica*, while the tegument, tegumental cells, and parenchymal cells were not stained. Sandwich ELISA showed the number of sera examined and the number and percentages designated positive for day 1 to 35 of infection. The mean optical density (OD) from infected mice sera were significantly different from those of control sera at day 1, 4, 7 ($p < 0.05$), 21 and 35 ($p < 0.01$) post infection. Detectable antigen levels in sera peaked at day 35 post infection. Therefore, this method exhibited an accuracy of 98.5% of experimently infected mice and of 98.9% of naturally infected ruminants.

Keywords: *Fasciola gigantica*; cathepsin L; monoclonal antibody; cross-reaction; sandwich ELISA

บทคัดย่อ

รหัสโครงการ : MRG5580012

ชื่อโครงการ : การพัฒนาชุดทดสอบสำหรับตรวจวินิจฉัยโรค fasciolosis จากการติดเชื้อพยาธิใบไม้ในตับ *Fasciola gigantica* แบบ sandwich ELISA โดยใช้โมโนโคลนัลและโพลีโคลนัลแอนติบอดีต่อโปรตีน Cathepsin L

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การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อผลิต ศึกษาคุณลักษณะ และการทำให้บริสุทธิ์ของโมโนโคลนัลแอนติบอดี (MoAb)และโพลีโคลนัลแอนติบอดี (PoAb) ที่มีความจำเพาะต่อ recombinant cathepsin L (rCatL) ของพยาธิใบไม้ตับ *Fasciola gigantica* และใช้ประโยชน์จากแอนติบอดีดังกล่าวในการศึกษาตำแหน่งการกระจายตัวของแอนติเจนชนิดนี้และใช้ประโยชน์ในการพัฒนาวิธีการตรวจสอบการติดเชื้อในสัตว์ที่เป็นโรค Fasciolosis ไฮบริโดมาที่ผลิตโมโนโคลนัลแอนติบอดีต่อ rCatL ได้มาจากการทำปฏิกิริยารวมตัวกันของเซลล์ myeloma และเซลล์จากม้ามของหนูทดลอง ซึ่งสามารถตรวจคุณสมบัติของโมโนโคลนัลแอนติบอดีดังกล่าวได้ด้วยวิธี indirect ELISA และ EITB เพื่อนำมาศึกษา cross-reaction และ การกระจายตัวของแอนติเจนในเนื้อเยื่อระยะต่าง ๆของพยาธิใบไม้ตับโดยใช้วิธี indirect immunoperoxidase แอนติบอดีที่มีความบริสุทธิ์ถูกนำมาใช้ในการใช้ในการพัฒนาวิธีการตรวจสอบการติดเชื้อในสัตว์ที่ติดโรคทั้งในห้องทดลองและ ติดโรคตามธรรมชาติด้วยวิธี sandwich ELISA ผลการศึกษาในครั้งนี้พบว่าโมโนโคลนัลแอนติบอดีที่ผลิตได้มีความจำเพาะสูงต่อ rCatL รวมถึงมีความจำเพาะสูงต่อ native CatL ในสารที่สกัดได้จากทั้งตัวพยาธิและสารคัดหลั่งจากพยาธิ และมีความจำเพาะสูงต่อ native CatL นอกจากนี้แล้วจากการศึกษาไม่พบ cross-reaction กับแอนติเจนของพยาธิใบไม้ พยาธิตัวตืด และพยาธิตัวกลมชนิดอื่นๆ จากการศึกษาพบว่า CatL มีการกระจายตัวมากอยู่ที่บริเวณเยื่อบุผิว caeca และ lumen ของ caeca ของเมตาเซอร์คาเรีย ตัวอ่อนระยะแรก ตัวอ่อนอายุ 1, 3 ,5 สัปดาห์ และตัวเต็มวัยของพยาธิใบไม้ในตับ *F. gigantica* จากการศึกษาด้วยวิธี sandwich ELISA พบว่าสามารถตรวจพบ CatL จากซีรัมของหนูทดลองตั้งแต่วันที่ 1 - 35 ของการติดโรค ค่า OD เฉลี่ยของหนูทดลองที่ติดโรคมีความแตกต่างอย่างมีนัยสำคัญ เมื่อเปรียบเทียบกับค่า OD เฉลี่ยของหนูทดลองที่ไม่ติดโรคหรือกลุ่มควบคุม ดังนี้ วันที่ 1, 4, 7 ($p < 0.05$), 21 และ 35 ($p < 0.01$) ระดับของแอนติเจนในซีรัมขึ้น peak ณ วันที่ 35 หลังการติดโรค ค่าความแม่นยำในการตรวจสอบเท่ากับ 98.5% ในหนูทดลองและ 98.9% ในสัตว์เคี้ยวเอื้องที่ติดโรคจากธรรมชาติ

คำหลัก: พยาธิใบไม้ตับ *Fasciola gigantica*; cathepsin L; โมโนโคลนัลแอนติบอดี; cross-reaction; sandwich ELISA

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LIST OF ABBREVIATIONS

AEC	=	3-Amino-9-ethylcarbazole
BSA	=	Bovine serum albumin
°C	=	Degree Celsius
CFA	=	Complete Freund's adjuvant
cm	=	Centimeter
cm^2	=	Square centimeter
Da	=	Dalton
DMSO	=	Dimethyl sulfoxide
DPI	=	Days post infection
EDTA	=	Ethylenediamine tetraacetic acid
EITB	=	Enzyme-linked immunotransfer blot
ELISA	=	Enzyme-linked immunosorbent assay
ES	=	Excretory-secretory
FCS	=	Fetal calf serum
g	=	Gram of force of gravity
h	=	hour
HAT	=	Hypoxanthine-aminopterine-thymidine
H_2O_2	=	Hydrogen peroxide
HRP	=	Horseradish peroxidase
IFA	=	Incomplete Freund's adjuvant
Ig	=	Immunoglobulin
kDa	=	Kilodalton
L	=	Liter
µg	=	Microgram
µl	=	Microliter
µm	=	Micrometer
M	=	Molar
MoAb	=	Monoclonal antibody
mg	=	Milligram
min	=	Minute
ml	=	Milliliter

LIST OF ABBREVIATIONS (CONT.)

mM	=	Millimolar
MW	=	Molecular weight
N	=	Normality
NaCl	=	Sodium chloride
Na_2CO_3	=	Sodium carbonate
$NaHCO_3$	=	Sodium bicarbonate
NEJ	=	Newly excysted juvenile
nM	=	Nanometer
PAGE	=	Polyacrylamide gel electrophoresis
PBS	=	Phosphate buffered saline
PEG	=	Polyethylenegylcol
PI	=	Propidium iodide
PoAb	=	Polyclonal antibody
PMSF	=	Phenylmethylsulfonyl fluoride
rpm	=	Round per minute
RPMI	=	Roswell Park Memorial Institute
sec	=	Second
SDS	=	Sodium dodecyl sulfate
TBS	=	Tris-buffer saline
TEM	=	Transmission electron microscopy
TEMED	=	N,N,N',N'-Tetramethylethylenediamine
Tris	=	Tris-(hydroxymethyl)-aminomethane
v/v	=	Volume by volume
w/v	=	Weight by volume

CHAPTER I

INTRODUCTION

Fasciolosis is a major disease of ruminants and is responsible for serious economic losses from reduced production of cattle, buffaloes, sheep and goats throughout the world, including Thailand. In the tropics, the major parasite is *F. gigantica*. The current method for diagnosis of fasciolosis in ruminants is based on the microscopic detection of eggs in feces. Although demonstration of circulating antibodies has been used for epidemiological studies, the presence of antibodies is not the direct indicator of active infection, and cross-reactivity with other parasites is often difficult to differentiate. The detection of the circulating antigens rather than antibodies is considered to be a more reliable method for evaluating the status of infection, which could be used to monitor the efficacy of treatment, and the effectiveness of future candidate vaccines. The method of circulating antigen detection has been used successfully in the diagnosis of many parasitic diseases.

In my previous research project **(MRG5280059)** (Anuracpreeda et al., 2011), I have produced, characterized and purified the monoclonal antibody (MoAb) and polyclonal antibody (PoAb) specific against recombinant cathepsin B3 (rCatB3) of *Fasciola gigantica* and used both antibodies to study the distribution of these antigens and application in immunodiagnosis of fasciolosis in animals. Results revealed that MoAbs showed stronger reaction with rCatB3. In addition, these MoAbs also exhibited strong reaction with native CatB3 in WB fraction of Metacercariae (Met) and newly excysted juvenile (NEJ). Moreover, in the cross-reactivities studies, no reaction was detected in the WB antigens from other trematode and nematode parasites as compared to that from *F. gigantica*. Immunolocalization of CatB3 in metacercariae, NEJ, 4-week-old juvenile and adult *F. gigantica* performed by immunoperoxidase technique by using these MoAbs as probes indicated that CatB3 was present in high concentration in the caecal epithelium and caecal lumen of the Met and NEJ, but not in the 4-week-old juvenile and adult fluke. Purified specific antibodies against rCatB3 of *F. gigantica* were used to develop both sandwich ELISA and tested for CatB3 in sera of experimentally infected mice as well as naturally infected ruminants. Sandwich ELISA showed the number of sera examined and the number and percentages designated positive for day 1 to 35 of infection. The mean optical density (OD) from infected mice sera were significantly different from those of control sera at day 1, 4, 7, 21 and 35 ($p < 0.05$) post infection. Detectable antigen levels

in sera peaked at day 1 post infection. Therefore, this method exhibited an accuracy of 93.75% of experimently infected mice and of 99% of naturally infected ruminants. However, CatB3 is the antigens released and function in newly excysted juvenile and very young parasites. Hence, its assay could be used for detection of early infection. In chronic and late infection, where mature parasites are lodged in bile ducts of the liver and gall bladder, the immune detection of CatB3 may not sensitive enough. Hence, there is still a need for an immunodetection system for late and chronic infection of *F. gigantica*.

Cathepsin L (CatL) was found to be a major circulating antigen released from the adult parasite during its course of infection. Therefore, in this research, I have focused on the production and characterization of monoclonal antibody (MoAb) and polyclonal antibody (PoAb) specifically against recombinant Cathepsin L (rCatL) of *F. gigantica* and used them in the sandwich ELISA method, which was developed for detecting the circulating parasite antigens in sera of experimentally and naturally infected animals. Hopefully, this method of detection could increase the sensitivity and specificity for the immunodiagnostic assay for fasciolosis, especially for the chronic and patent infection with *F. gigantica*.

CHAPTER II

OBJECTIVES

Overall objective: The overall objective of this project is to produce, characterize and purify the MoAb and PoAb specific against rCatL of *F. gigantica* and use these antibodies for devising the immunodiagnostic method for fasciolosis.

Specific objectives: To achieve the overall objective, there are 3 specific objectives that will be performed in steps as follows:-

1. To produce and characterize MoAb and PoAb specifically against rCatL of *F. gigantica*.
2. To purify the MoAb and PoAb against rCatL of *F. gigantica*.
3. To develop a monoclonal antibody (MoAb)-based sandwich enzyme-linked immunosorbent assay (sandwich ELISA) for the detection of circulating CatL in serum of experimentally and naturally infected animals with fasciolosis caused by *F. gigantica*.

CHAPTER III
LITERATURE REVIEW

1. Fasciolosis

Fasciolosis is a major disease of ruminants and is responsible for serious economic losses from the reduced production of cattle, buffaloes, sheep and goats throughout the world. The disease is caused by liver flukes of the genus *Fasciola*, of which *Fasciola hepatica* and *Fasciola gigantica* are the most common representatives (World Health Organization, 1995). *F. gigantica* infection of animals is found commonly in tropical regions of the world. Countries where *F. gigantica* infections are reported include those in Africa, Asia, many Pacific islands including Hawaii (where it has also been reported in human), the Middle East and Southern Europe, the south of the USA, as well as Thailand (Fabiyi, 1987). The disease is characterized by the destruction of the host's liver tissue and damage to the bile ducts. The major symptoms found in ruminants are weakness, loss of body weight, slow growth rate, anemia and death in severe infections, especially in young animals (Dargie, 1987; Behm and Sangster, 1998; Phonpark and Srikitjakara, 1989; Srihakim and Pholpark, 1991; Sukhapesna et al., 1994). The economic importance of the disease has been estimated to cause worldwide losses to the livestock industry of more than 3 billion U.S. dollars per annum (Spithill et al., 1999). Human infection with liver flukes is also recognized by the World Health Organization as an emerging human health problem, with more than 500 million people at risk of infection with *Fasciola*, *Opisthorchis*, or *Clonorchis* (Hillyer and Apt, 1997; Spithill et al., 1999; Mas-Coma et al., 2005). There are at least 2.4 million people infected with *Fasciola*, and infection rates in children of up to 72% have been observed in Bolivia (Mas-Coma et al., 2005). The clinical manifestations in human infection include fever, right hypochondriac pain, persistent diarrhea and vomiting. The severity of the *Fasciola* infection of animals and humans depends primarily on the number of viable metacercariae (infective stage of parasite) being ingested and their infectivity (Arjona et al., 1995). In Thailand, fasciolosis is caused by *F. gigantica* and has been reported to have the highest incidents in the northeastern and northern provinces (Sukhapesna et al., 1994). The economic losses in Thailand are estimated to be at least 350 million bahts (10 million $US) per year (Srihakim and Pholpark, 1991). The prevalent rate of infection in Thailand varies from 4-24% in cattle and buffaloes, with the highest incidents of infection in the northeast (up to

85% of cattle and buffaloes) (Phonpark and Srikitjakara, 1989; Srihakim and Pholpark, 1991; Sukhapesna et al., 1994).

2. Life cycle of *Fasciola gigantica*

The life's cycle of *F. gigantica* is quite complex. It begins when the enormous number of immature eggs being passed from the common bile duct into the duodenum, and subsequently into the feces of mammalian hosts. The egg can undergo embryonation outside the host, which is influenced by temperature, humidity, oxygen tension and pH range. Hatching of miracidium is stimulated by light and temperature (Andrews, 1999). Once hatched from the eggs, the motile miracidium become active and need to search for and invade into the suitable snail intermediate hosts. The amphibious snails belonging to the genus *Lymnaea*, with different species having different affinities for the parasite. In Thailand, *Lymnaea (Radix) auricularia rubiginosa* is the principal intermediate host, whilst in Nepal and Pakistan the most important species is *L. auricularia rufescens*. In the snail, the parasites undergo several developmental stages. After it penetrates the snail's body wall, shedding its ciliated cells, it transforms into saclike sporocyst. One sporocyst contains a number of germ balls, which develops to new larval form, redia. Multiplying redia breaks the sporocyst and typically move to hepatopancreas of snail host. Eventually, the development proceeds to the final stage in the intermediate host, the cercaria. The free-living cercariae are released from the snails in motile forms. On emerging from the snails, a cercaria attaches to submerged blade of grass or other vegetation (e.g. watercress), the tail falls away and the cercarial body secretes a four-layered cyst-covering from cystogenous glands present on the lateral regions of its body. The formation of this cyst wall may take up to two days, after which the metacercaria is fully infective to the definitive host. Development of metacercariae from miracidium takes a minimum of 6-7 weeks. The metacercarial cyst is only moderately resistant, not being able to survive dry conditions. If, however, they are maintained in conditions of high humidity and cool temperatures they may survive for up to a year. It has been reported that an infection of a snail with one miracidium can produce over 600 metacercariae (Morel and Mahato, 1987; Nobel et al., 1989; Charoenchai et al., 1997).

For the hosts to be infected, metacercariae, which attach on the grass blades, are ingested by the definitive hosts which are most commonly ruminants, such as cattle, buffaloes, sheep and goat. Within an hour after ingestion, the metacercaria excysts in the small intestine, releasing the young parasite (newly excysted juveniles or NEJ). The

excystment process is complex and involved two phases: 1) passive activation phase is the prerequisite for the newly excysted juvenile (NEJ) to emerge. This activation occurs in the stomach or rumen, and needs elevated temperature, reducing conditions, decreased pH, increased pCO_2, and the presence of bile salt. 2) Active emergence phase is triggered by bile and enzyme secreted by the parasite. Various hypotheses have been proposed for bile-initiated mechanism, such as muscular movements of the young fluke. After the emerging in the small intestine, NEJ rapidly penetrate through the intestinal wall and move to the peritoneal cavity. From here it migrates over a period of about seven days directly to the liver. The juvenile fluke then penetrates the liver tissues, through which it migrates, feeding mainly on blood for about six weeks. After this period, the fluke enters the bile duct, maturing to the fully mature adult about 3 months after initial infection. Egg production then commences, completing the life cycle (Kendall and Parfitt, 1953). In human, maturation from metacercariae into adult flukes takes approximately 3 to 4 months. The adult flukes (*F. gigantica*: up to 75 mm) finally reside in the large biliary ducts of the liver and gall bladder of mammalian hosts (Parichatikanond and Sarasas, 1984).

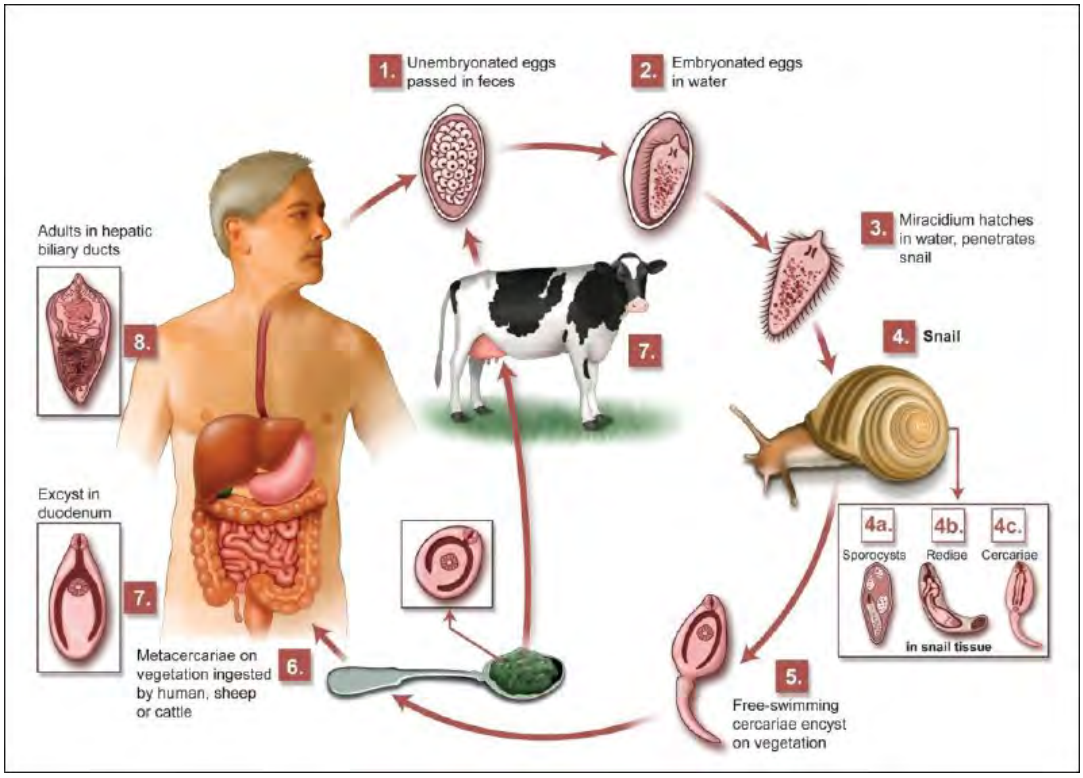


Figure 1 Life cycle of *Fasciola* species (http://www.clinicalimagingscience.org).

3. Candidate immunodiagnostic antigens

3.1 Cathepsin L (CatL)

The major proteases secreted by adult *Fasciola* belong to the family of cathepsin L-like proteases. In adult *F. hepatica*, two major forms of cathepsin L which are isolated and characterized can be divided into cathepsin L1 (Smith et al., 1993) and cathepsin L2 (Dowd et al., 1994). Cathepsin L1 and cathepsin L2 have been purified and appeared at molecular weight of 27 and 29.5 kDa as observed by SDS-PAGE. It has been reported that cathepsin L1 can cleave immunoglobulin at the hinge region and cathepsin L2 can cleave fibrinogen in a manner that led to the formation of fibrin clots (Dowd et al., 1995). Moreover, cathepsin L2 differed from cathepsin L1 and all others cathepsins in that it can cleave substrates with the proline residue in the P2 position. Besides these substrate specificities, cathepsin L1 and L2 can also degrade extracellular matrix and basement membrane components, such as collagen, fibronectin and laminin (Berasain et al., 1997). The liver fluke cathepsin Ls are active in neutral to alkaline conditions which might be due to the modification of certain proline residues to 3-hydroxyprolines while mammalian cathepsin L was active at the acidic pH of lysosomes. Histochemical studies also showed the presence of cathepsin L in the Mehlis' gland in adult *F. hepatica* which led to the suggestion that this enzyme is somehow involved in the process of eggshell formation (Wijffels et al., 1994; Spithill et al., 1997).

In *F. gigantica*, proteolytic enzymes from the ES products of adult worms were characterized as cysteine proteases and ranged in molecular sizes from 26 to 193 kDa on gelatin substrate PAGE. However, the most major bands were 26-28 kDa and displayed immunoglobulin degradating activity after partial purification (Fagbemi and Hillyer, 1992). Immunolocalization studies revealed that cathepsin Ls are synthesized and packaged in vesicles within the gut epithelial cells of *Fasciola* sp. (Yamasaki et al., 1992; Meemon et al., 2010). This corresponded with RNA *in situ* hybridization results which also showed the localization of cathepsin L RNA within the gut epithelial cells (Grams et al., 2001). Yamasaki et al. (2002) reported that multiple genes encoding cathepsin Ls in *F. gigantica* was analyzed by southern blotting with an estimated copy number of 10 closely related genes. Reverse transcriptase PCR using oligonucleotide primers based on sequences from cathepsin Ls of *F. hepatica* were used to clone cDNAs encoding cathepsin L-like proteinases from *F. gigantica*. Six different cathepsin L cDNA sequences were isolated and termed cathepsin L-A to L-F. They had sequence identity values ranging from 87-99% to the nucleotide sequences of the homologous genes from *F. hepatica*. The most

abundantly expressed from, cathepsin L-A, was produced in *E. coli* as recombinant protein (rFgCatL-A) and used as target for production of monoclonal antibodies. In the other study, recombinant *F. gigantica* procathepsin L, produced in yeast, showed that it was processed to an enzymatically active cathepsin L by pH-dependent autocatalysis while the pro-peptide deleted cathepsin L showed no enzyme activity, indicating that pro-region of *F. gigantica* procathepsin L is essential for the folding and/or refolding of functional cathepsin L (Yamasaki et al., 2002).

4. Strategies for the developments of diagnosis of *Fasciola* infection

The diagnosis for fasciolosis was usually performed by fecal examination for the parasite eggs. This frequently fails to detect the infection or misdiagnose the disease, because of the misjudgment of eggs in the feces as well as the missing of eggs during the early stage of infection when eggs have not yet been produced. Hence, it was necessary to find a more reliable and convenient diagnostic assay. It was believed that immunodiagnosis would be a better method, but no such method had been devised. The immunodiagnosis could be devised performed in three ways: detection of host's antibodies against *Fasciola* antigens (serodiagnosis), detection of *Fasciola* antigens in the host's blood circulation, and detection of *Fasciola* antigens in the host's feces.

Up to now, the main source of potential immunodiagnostic antigens in fasciolosis are the metabolic antigens released from the tegument and/or the excretion-secretion (ES) material of the parasites. The detection of the antibody against *Fasciola* antigens is used to predict the infection of the *Fasciola* parasite. The antigens in the excretory-secretory (ES) products of adult worms are first used for evaluating the infection. In *F. hepatica* infection, infected rats were shown to produce antibodies against ES products of adult flukes as early as the first week after the infection (Poitou et al., 1992). For practical application, many fractions of ES products have been used for diagnosis of fasciolosis. Rivera et al. (1988) tested a fractionated *F. hepatica* ES components at the molecular weight of 150 to 160 kDa and found that they were very reactive with sera from early fascioliasis cases of *F. hepatica*.

The potential of cathepsin L1 for immunodiagnosis has been proven by many studies. The recombinant cathepsin L-like protease generated by expression of the cDNA from adult *F. hepatica* in *Saccharomuces cerevisiae* was used in ELISA to assess the sera from sheep and calves experimentally or naturally mono-infected with *F. hepatica* and other parasites. The sensitivity of the cathepsin L ELISA for sheep and cattle sera

was 99.1 and 100%, respectively (Cornelissen et al., 2001), and the specificity was 98.5% for cattle sera and 96.5% for sheep sera. Specific and sensitive diagnosis of human fasciolosis using cathepsin L1, purified as a 27 kDa antigen from *F. gigantica* ES product (FG27), was performed (Maleewong et al., 1999). Currently, they partially sequenced the FG27 antigen and revealed the sequence of the 20 amino acids from the N- to the C-terminus (Tantrawatpan et al., 2005). The sequenced data proved to be homologous with the deduced amino acid sequence of the *F. gigantica* cathepsin L1 (Grams et al., 2001) (GenBank accession number AF112566), cathepsin L1-B (Grams et al., 2001) (GenBank accession number AF239264), and cathepsin L1-E (Grams et al., 2001) (GenBank accession number AF239267). Serologic tests for the diagnosis of fasciolosis have been developed as standard assays using authentic *F. hepatica* cathepsin L1 (O'Neill et al., 1998, 1999; Strauss et al., 1999; Rokni et al., 2002) or recombinant cathepsin L1 (O'Neill et al., 1999; Carnevale et al., 2001; Cornelissen et al., 2001) as marker antigens as well as a selected B-cell epitope (specific peptide 7; acetyl-DKIDWRESGYVTEVKDQGNC-carboxamide [peptide V]) from *F. hepatica* cathepsin L1 (Cornelissen et al., 1999, 2001). In addition, the recombinant *F. gigantica* cathepsin L1 (rCTL1) was used as a diagnostic reagent for human fascioliasis based on a cystatin capture enzyme-linked immunosorbent assay (ELISA) (Tantrawatpan et al., 2005). In 2003, Rokni and co-workers compared the efficacy of somatic antigens and cysteine proteinase of *F. gigantica* in detecting serum IgG by ELISA to diagnose human fasciolosis. The cut-off point for somatic and cysteine proteinase antigens was 0.40 and 0.35, respectively. The positive and negative predictive values of cysteine proteinase antigen were 98 and 100%, respectively. More recently, two fractions of cysteine proteinases, with molecular weights of 28 (P28) and 34 kDa (P34), have been used in ELISA tests, and showed a rapid and consistent detection of specific IgG in all experimentally goats infected with *F. hepatica* (Ruiz et al., 2003). The IgG response to P28 was the first to be detected as early as 2 to 3 weeks post infection and remained elevated throughout the experiment. The response to P34 was detected later (4-6 week post infection) and disappeared in some animals at 18 week post infection, while flukes were still present in the bile ducts. All of these studies have confirmed the potential of cathepsin L for immunodiagnosis for fasciolosis.

The detection of the parasite antigens that persist in the host's blood circulation is the more direct method for diagnosing whether the living parasites are still in the hosts, as well as to give an estimate of the parasite burden. Few studies have been done on the immunodiagnosis of fasciolosis by the detection of circulating antigens. For antigen detection, the sandwich ELISA is the most commonly used method. For example,

Viyanant and colleagues (Viyanant et al., 1997) could produced a monoclonal antibody (MoAb) that reacted with 66 kDa surface tegumental (ST) antigen of adult worms of *F. gigantica*, and this MoAb was used to detect circulating antigen in sera of experimentally and naturally infected cattle by sandwich ELISA. The circulating antigen of *F. gigantica* was demonstrated in the sera of all experimentally infected animals as early as the first week after the infection, and it remained detectable until the experiment was terminated at week 32 post infection. Unfortunately, the hybridoma clone was not stable. In 2004, Velusamy and colleagues (2004) showed by using sandwich ELISA that the circulating 54 kDa and whole worm antigen of *F. gigantica* were detected in the serum samples of experimentally infected calves as early as 2 weeks post infection and persisted until the end of experiment at 26th week post infection. However, monoclonal and polyclonal antibodies developed against these two antigens were either unstable or unable to capture the corresponding antigens in the host circulation, and the cross reaction with antigens from other parasites has also been shown in these assays. Recently, immunodiagnosis has focused on the detection of antigens in the feces of infected hosts, since the parasite releases metabolic products, ES antigens, and somatic antigens into the feces. Dumenigo and co-workers (1996) used sandwich immunoassay based on monoclonal antibody for the detection of *F. hepatica* antigen in the feces of 100 cattle infected with *F. hepatica,* 56 animals with parasitologically proven infections with other parasites and 100 uninfected animals. It was found that the *Fasciola* antigens were detected in all the fecal samples from animals with fasciolosis, but none of the samples from the uninfected animals or from those with other parasitic infections had significant levels of *F. hepatica* antigens. Moustafa and colleagues (1998) used sandwich ELISA for the detection of *F. hepatica* antigens in stool sample of experimentally infected mice, rats and rabbits over a period of 18 weeks using affinity-purified polyclonal antibodies raised against the whole adult *F. gigantica*. The test demonstrated fascioliasis in mice earlier than in rats or rabbits (4, 6 and 7 weeks post infection, respectively). Abdel-Rahman and co-workers (1998) used hyperimmune rabbit antiserum raised against the purified 26 to 28 kDa glycoprotein to detect the coproantigen in the feces of 27 experimentally infected calves with known numbers of flukes using monoclonal antibody against this 26 to 28 kDa coproantigen in enzyme-linked immunosorbent assay, and found that this assay could identify all calves infected with more than 10 flukes, and as little as 300 pg of coproantigen / ml of fecal supernatant could be detected. Estuningsih et al. (2009) generated monoclonal antibodies to native cathepsin L and used them to to capture this antigen in feces of infected cattle. The results showed that the sensitivity and specificity of

coproantigen detecting ELISA (95 and 91%, respectively) was better than the anti–*F. gigantica* antibody ELISA (91 and 88%, respectively) and to fecal egg counting (87 and 100%, respectively). However, feces is difficult to collect from individual animals, and inconvenient as well as unsavory to perform the test. Immunoassay for circulating antigens in the individually collect blood sample will be more convenient.

CHAPTER IV
MATERIALS AND METHODS

The materials and methods were divided into 3 sections which correspond to the 3 objectives described in Chapter 3.

1. Production of MoAbs and PoAbs against rCatL antigens of *F. gigantica*

1.1 Specimen collections

1.1.1 Metacercariae

F. gigantica metacercariae were obtained from infected *Lymnaea ollula* snails. The snails were be infected with miracidiae and allowed to develop sporocysts and cercariae. The cercariae were shed from the snails and settled on the plastic sheets, on which cercariae encysted and developed into metacercariae. After about 8-10 weeks, metacercariae were observed under stereomicroscope, brushed from the plastic sheet and washed several times with normal saline solution (NSS) and kept at 4 °C until use.

1.1.2 Newly excysted juveniles (NEJ)

NEJ were collected by excystment of metacercariae and washed several times before being processed for further experiment.

1.1.3 Juvenile parasites

Juveniles *F. gigantica* were obtained from infected male Golden Syrian hamsters, each of which was previously infected orally with 30 metacercariae of *F. gigantica*. The juveniles wer recovered by teasing the liver tissues isolated from infected hamsters, and the recovered parasites were washed several times with normal saline solution (NSS) before being used in further studies.

1.1.4 Adult parasites

Adult *F. gigantica* and *F. hepatica* were collected from gall bladders and intrahepatic bile ducts, while other trematodes (*Gigantocotyl explanatum*, *Eurytrema pancreaticum, Paramphistomum cervi, Cotylophoron cotylophorum, Fischoederius cobboldi and Gastrothylax crumenifer*) were collected from bile ducts, pancreas, rumen and reticulum as well as *Schistosoma spindale* were collected from mesenteric vein. Cestodes (*Moniezia benedeni and Avitellina centripunctata*) were collected from intestine. Nematodes (*Trichuris* sp., *Haemonchus placei* and *Setaria labiato-papillosa*) and were collected from abomasum and peritoneal cavity of infected cattle or water buffaloes killed at the local abattoirs.

Adult *Schistosoma spp* (*Schistosoma mansoni, Schistosoma japonicum and Schistosoma mekongi*) were obtained by perfusion of mice, 8 weeks after infection with schistosome cercariae. Adult *Opisthorchis viverrini* were collected from adult golden Syrian hamsters (140-160 g) infected orally with 50 to 100 metacercariae obtained from the muscles of naturally-infected cyprinoid fishes. After 4 weeks of infection, the adult worms were obtained by teasing the liver tissues.

All parasite samples were washed several times with normal saline solution (NSS) to remove the host blood, bile and contaminating microorganisms before being processed for further experiments.

1.2 Antigen preparation

1.2.1 Recombinant CatL antigens of *F. gigantica*

I have re-expressed cDNA of CatL constructed earlier in our lab (Meemon et al., 2010) in yeast using the pFLAG expression system, and then isolated the recombinant proteins (rCatL) from the culture supernatants. The recombinant proteins were expressed with a poly-histidine tag at the C-terminus to allow affinity purification on a nikle-chelated column.

1.2.2 Whole body (WB) antigens of metacercarial, juvenile and adult parasites

Whole parasites (all stages of *F. gigantica*, adult *F. hepatica* and other species) were homogenized in lysis buffer and extracted overnight with continuous rotation at 4 °C. The suspension was centrifuged at 5000 x *g*, 4 °C, for 20 min to get rid of eggs, and the supernatant was collected and lyophilized (Anuracpreeda et al., 2008, 2013b). Protein concentration was determined by a modified Lowry's method (Lowry et al., 1951). These extracts were stored at -70 °C until use in subsequent experiments.

1.2.3 Tegumental antigens (TA) of adult *F. gigantica*

To obtain TA, the method described by Anuracpreeda et al. (2006) was used. Briefly, adult worms were extracted with a non-ionic detergent for 20 min at room temperature. Shedding of the tegument was monitored under a light microscope, and at the end of extraction the supernatant was collected and devoided of contaminating eggs by centrifugation at 5000×*g* for 20 min. The supernatant that contained soluble TA was collected and dialyzed overnight at 4 °C, using Spectra/Por dialysis membrane (Spectrum Medical Industries, Los Angeles, California, USA.). The protein concentration was

determined by Lowry's method (1951), and the protein solution was stored at −70 °C until use in later experiments.

1.2.4 Excretory-secretory (ES) antigens of adult *F. gigantica*

ES antigens were prepared by incubating newly collected, living adult *F. gigantica* in culture media for 3 h at 37 °C. After incubation, the culture medium was centrifuged at 5,000 x g for 20 min at 4 °C to remove the parasites' eggs from the culture medium. The supernatant was then filtered through 0.22 µm Millipore filter. The protein concentration was measured by Lowry's method (1951) and stored at -70 °C until further use.

1.3 Immunization with rCatL antigens

Eight-week-old BALB/c mice were subcutaneously immunized with 10 µg of recombinant proteins in 200 µl complete Freund's adjuvant and followed by subcutaneous injection with the same dose in 200 µl incomplete Freund's adjuvant on day 21. The last immunization was performed 3 week later by intravenous injection with 20 µg of the antigens in 100 µl 0.1M PBS. Three ELISA were performed to determine the antibody titer in the immunized mouse serum. Mice that show the highest positive ELISA titer against the antigens were sacrificed and its spleen removed for fusion experiment to produce monoclonal antibody.

1.4 Production and screening of MoAbs

MoAbs specific against rCatL antigens were obtained by fusion of recombinant proteins-immunized spleen cells with mouse myeloma cells. The positive clones were selected and expanded according to Anuracpreeda et al. (2011). MoAbs produced by clones of hybridoma were screened by indirect ELISA. Briefly, 100 µl of 2.5 µg/ml each antigens of *F. gigantica* diluted in coating buffer, pH 9.6 were added into each well of polystyrene microtiterplate (Nunc A/S, Roskilde, Denmark) and incubated at 37 °C overnight. Excess antigens and unbound material were removed, and the plate was washed three times with distilled water. Each time the washing fluid is left in the wells for approximately 1 min. Consequently, the plate was tapped dry and blocked with blocking solution (0.25 % bovine serum albumin (BSA), 0.05% Tween 20 (Sigma Co.) in 0.01 M PBS, pH 7.2) 100 µl/well at 37 °C for 30 min, and then similarly wash prior to adding 100 µl of the hybridoma supernatant to be tested at 37 °C for 2 h. The plate was washed as above before goat anti-mouse immunoglobulin conjugated with peroxidase was added to

each well at 100 µl, and subsequently incubated for 1 h at 37 °C, and washed again. After excess conjugated was discarded, 100 µl of 3, 3', 5, 5'-tetramethyl benzidine (TMB) substrate was added and the enzymatic reaction was allowed to take place for 5 min at room temperature. Finally, the enzymatic reaction was stopped by addition of 100 µl of 1N HCl. The optical density was measured in a microplatereader. Specific MoAb class and subclass were determined by enzyme immunoassay using the SBA Clonotyping™ System/HRP (SouthernBiotech, Birmingham, USA).

1.5 Immunoblotting and cross reactivities studies

The method of horizontal electrophoretic transfer using Multiphor II Nova Blot unit (Pharmacia LKB Biotechnology, Sweden) which was described by Anuracpreeda et al. (2008, 2013a) was used in this research. Briefly, recombinant CatL protein antigens were separated by SDS-PAGE according to a standard protocol and electroblotted onto the nitrocellulose membrane. Afterthat, the nitrocellulose membrane was cut into strips and washed with 0.1 M Tris-Cl, 0.15 M NaCl pH 7.4 for 5 min. Non-specific binding was blocked with blocking solution (5 % skim milk in 0.1 M TBS, pH 7.4) at room temperature for 2 h, and incubated in culture fluid of the positive MoAb at room temperature for 2 h. Thereafter, the strip was washed three times with 0.1 M TBS, pH 7.4 at room temperature for 5 min each. And then, the peroxidase-conjugated goat anti-mouse immunoglobulin diluted 1:4000 in 1 % skim milk in 0.05 M Tris-Cl, 150 mM NaCl, pH 7.4 was added to the strip and incubated at room temperature for 1 h. The reaction was detected by addition of the specific enzyme substrate 3, 3', 5, 5'-tetramethyl benzidine (TMB) for 1-5 min or until band appears at room temperature. Finally, the enzyme reaction was stopped by adding distilled water. The sera of naturally infected cow (CIS) and fetal calf (FCS) were also used as primary antibodies to serve as positive and negative controls. For detecting CIS conjugated antigens, peroxidase-conjugated rabbit anti-bovine immunoglobulin was used as the secondary antibody.

For the cross reactivities studies, WB antigens from *F. gigantica* and other trematode and non trematode parasites were separated and blotted with various MoAbs by similar method as mentioned above. In addition, indirect ELISA was also used to confirm and quantify the degree of the cross reactions. MoAb and antigens which show the least cross reactions against other parasites antigens were tested against immune sera of naturally infected cattle collected in fields from various part of Thailand in order to detect the circulating antigens and/or antibodies, in co-operation with Dr. Manoch

Boonrawd (Regional Bureau of Animal Health and Sanitary IV, Khonkaen), Dr. Poonsin Chairat and Dr. Nirute Srisroy (Kalasin Provincial Livestock Office, Department of Livestock, Ministry of Agriculture and Co-operatives, Bangkok), and Dr. Nonthasak Piempol (Pusing Research and Training Center, Rajamangala University of Technology, Isan Kalasin Campus, Thailand). From this experiment, the diagnostic potential of each antigen could be evaluated by specific MoAbs.

1.6 Immunolocalization of CatL

The immunoperoxidase technique described by Anuracpreeda et al. (2009a, 2011, 2014) was used to analyze the distribution and relative concentration of CatL in the paraffin sections of *F. gigantica* at each developmental stages (metacercariae, NEJ, 1, 3, 5-week-juveniles and adults) using seven MoAbs as probes. Briefly, the sections were deparaffinized and rehydrated through xylene and decreasing serial concentrations of ethyl alcohol (from 100% to 50%) for 3 min each before rinsing with tap water. The sections were incubated in 0.01 M citrate buffer, pH 6.0 in a microwave oven at 500-700 kV for 5 min, 3 times. Then the endogenous peroxidase in the tissues was quenched by treatment with 3% H_2O_2 diluted in tap water for 30 min, and washed with 0.01 M PBS containing 0.1% Tween-20 for 5 min, 3 times. Thereafter, non-specific binding was blocked by incubation in 0.1% (w/v) glycine in 0.01 M PBS pH 7.4 and 4% (w/v) BSA in 0.01 M PBS pH 7.4, for 30 min and 1 h, respectively. The sections were incubated in specific MoAbs against rFgCatL for 2 h at room temperature. After washing three times for 5 min each, the sections were incubated with biotinylated goat anti-mouse IgG (Zymed Laboratory Inc., South San Francisco, CA, USA), diluted to 1:200 in 0.01 M PBS, for 30 min at room temperature. Subsequently, they were incubated with HRP-conjugated streptavidin (Zymed Laboratory Inc.), diluted to 1:200 in 0.01 M PBS, for 30 min at room temperature, and then washed. The color reaction was developed by using AEC (3-amino-9-ethylcarbazole) substrate solution (Zymed Laboratory Inc.) in the dark. Finally, the reactions were stopped by adding tap water and the stained sections were mounted in Vectashield (Vector Laboratories Inc., Burlingame, CA, USA). The sections were observed and photographed under a light microscope (Nikon, Eclipse E600) equipped with a DXM 1200F digital camera.

1.7 Production of the PoAb against rCatL antigens of *F. gigantica*

PoAb against rFgCatL used for detecting the antigen captured by the immobilized MoAb was prepared by immunizing NewZealand white rabbits with rFgCatL. The animals were primed by subcutaneous injection on the shoulder region with 500 μg rFgCatL in 500 μl PBS solution emulsified with an equal volumeof complete Freund's adjuvant (Sigma–Aldrich Inc.). Three weeks later, the animals were given a booster injection of 250 μg rFgCatL in PBS emulsified in incomplete Freund's adjuvant (Sigma–AldrichInc.) via the same route. Three days before blood collection, the final boosting with 250 μg rFgCatL was given by the sameroute without adjuvant. The rabbits were bled, and the antibody titers in the antisera were tested by indirect ELISA against rFgCatL. Rabbit anti-sera against rFgCatL were purified by ammonium sulfate precipitation and ion-exchange chromatography for the next experiments.

2. Purification of the MoAbs and PoAbs against rFgCatL of *F. gigantica*

2.1 Purification of MoAbs and PoAbs

2.1.1 Ammonium sulfate precipitation

MoAb in culture supernatant and PoAb in serum were concentrated and purified by precipitation in saturated ammonium sulfate solution. Briefly, equal volume of ice-cold saturated solution of ammonium sulfate was added dropwise to the culture supernatant. The reaction was allowed to proceed overnight with gentle mixing. After centrifugation the mixture, the precipitate was collected, reconstituted to a required volume with saline, dialysed exhaustively against a large volume saline and finally followed by dialyzing against PBS. Protein content or dialysate was determined by measuring absorbance at 280 nm (Auracpreeda et al., 2009b, 2013a)

2.1.2 Gel-filtration and ion exchange chromatography

MoAb and PoAb were purified from the ammonium sulfate precipitated globulin fraction by a gel-filtration and ion exchange chromatography, respectively. Precycling equilibration and packing were carried out essentially. In brief, the column were packed. The globulin fraction obtained by ammonium sulfate precipitation and dialyzed against the buffer were applied to the column. Appropriate immunoglobulin fractions were collected and pooled. The protein content was estimated from the optical density (OD) value measured. The pooled immunoglobulin was dialyzed, lyophilized and kept at 4 $^{\circ}$C (Sirisinha et al., 1991; Auracpreeda et al., 2009b)

2.1.3 Biotin conjugation of PoAb

Rabbit IgG anti-TA was covalently conjugated with biotin using N-hydroxysuccinimidobiotin (Sigma Co.) derivatived by the method of Neruker et al. (1984) and Auracpreeda et al. (2009b, 2013a). Briefly, the purified immunoglobulin was dialyzed overnight, clarified by centrifugation and the protein solution was diluted to a final concentration. A biotin solution freshly prepared in DMSO was added immediately to the immunoglobulin. The reaction mixture was allowed to take place and was then exhaustively dialyzed to remove unconjugated biotin and DMSO. The supernatant was mixed with an equal volume of glycerol and stored at -20 $^{\circ}$C.

3. Development of a monoclonal antibody (MoAb)-based sandwich enzyme-linked immunosorbent assay (sandwich ELISA) for the detection of circulating CatL in serum of experimentally and naturally infected animals with fasciolosis caused by *F. gigantica*.

3.1 Experimental mice

A total of clinically normal 5-week-old male ICR 120 mice, *Mus musculus*, from National Laboratory Animal Center, Mahidol University, Thailand were used. Mice were randomly divided into two groups: control and treated groups. Sixty control and sixty treated mice were divided into 5 groups. They were sacrificed at 1, 4, 7, 21, and 35 days post infection. Control groups were received only 0.85% NaCl for 0.5 ml, and each mouse in the treated groups was orally infected with 15 metacercariae of *F. gigantica*. At necropsy, whole blood of the animals in both control and treated groups were collected into tubes without anticoagulant. For antigen and antibody detection, the sera were collected from whole blood by conventional method. For cross-reactivity study, the sera from other parasitic infection were performed. There are 10 sera from mice infected with *S. mansoni* and 10 sera from hamsters infected with *O. viverrini*. In addition, twenty sera from normal hamsters were used as negative control.

One hundred and twenty 5-week-old ICR mice, from NationalLaboratory Animal Center, Mahidol University, Thailand, were randomly divided into two groups: control and metacercarial infected groups, sixty control and sixty infected mice were subdivided into 5 subgroups (12 mice per group). They were sacrificed at 1, 4, 7, 21and 35 days post infection. Control groups received 0.5 ml of 0.85% NaCl solution and treated groups were orally infected with 15 *F. gigantica* metacercariae per mouse. At necropsy day, all infectedmice were anaesthetized and their peritoneal cavities were opened.The carcasses

and organs were carefully examined for any pathological changes and the presence of flukes. The livers were teasedand immersed in 0.85% NaCl solution and the flukes were recovered. Blood samples of all animals were collected aseptically into tubeswithout anticoagulant. For antigen and antibody detections, thesera were collected and separated by centrifugation of the whole blood. For cross-reactivity study, ten sera were collected frommice infected with *S. mansoni* and 10 sera from hamsters infected with *O. viverrini*. In addition, twenty sera from non-infected hamsters were collected and used as the negative control.

3.2 Naturally infected cattle and buffaloes

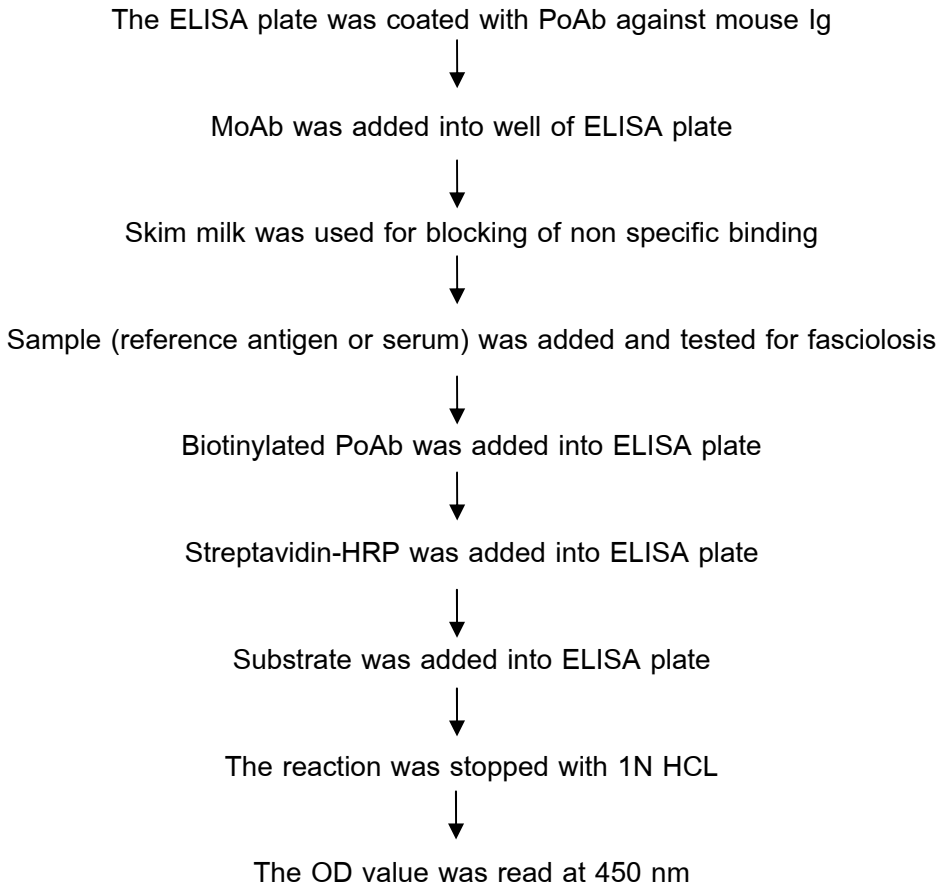
Sera from cattle infected with *F. gigantica,* other trematode, nematode parasites and uninfected cattle were collected fromfields in many regions of Thailand.

3.3 The specificity of sandwich ELISA and the lower detection limit

To determine the lower limit of detection, the rCatL and WB, TA, Es of *F. gigantica* were serially diluted in 1% BSA–0.05%PBST and analyzed by the sandwich ELISA. The optical density (OD) values were recorded and correlated with the amounts of antigen.The end point of detection limit was considered to be the lowestamount of antigen still giving the positive OD values. The specificity of sandwich ELISA was determined by using WB antigens from other trematode and nematode parasites. Each of these antigens was prepared at various concentrations and used to detectpossible presence of CatL antigen.

3.4 Detection of circulating CatL antigens by sandwich ELISA

The pre-coated antibody (PoAb against mouse Ig) was diluted in buffer to a final protein concentration and it was applied to each well of the microtiter plate. After incubation, the plate was washed. Then, the MoAb was diluted to a final concentration and it was applied to each well of the microtiter plate. After incubation, the plate was washed with the same washing buffer. After washing as previously described, appropriate concentrations of reference antigen and samples were added. After incubation, the plate was washed and biotinylated PoAb against rCatL was added. After washing, streptavidin-conjugated peroxidase was added and incubated. Then the plate was washed and substrate solution (1N HCL) was added. The enzymatic reaction was carried out and the reaction was stopped after 5 mins. The optical density was read at 450 nm. The detail was summarized below:



3.4 Statistical analysis

All data from the detection of CatL antigens in serum of experimentally and naturally infected animals with fasciolosis caused by *F. gigantica* were calculated and analyzed with independent-samples t-test or one-way repeated measures ANOVA. Each group was considered significant if (probabilities) *p*-value is statistically lower than 0.05 ($p < 0.05$). The diagnostic sensitivity, specificity, accuracy, false-positive and negative rate, and predictive values were calculated using the method of Galen (1980).

CHAPTER V

RESULTS

1. Production and Characterization of MoAb and PoAb specifically against rCatL of *F. gigantica*

In this study, MoAb and PoAb specifically against rCatL of *F. gigantica* have been already produced and characterized.

1.1 Immunization with rCatL antigens

Antibody titers of mouse serum against rCatL antigens of *F.gigantica* were tested by indirected ELISA technique. A mouse showing OD value higher than mean value of the pre-immune serum plus 3 standard deviations (SD) is considered to be the high responder. The high responder was used for fusion for production of MoAb as shown in Figure 2.

1.2 Production and screening of MoAbs

The hybridoma clones expressing MoAb against rCatL of *F. gigantica* were produced by fusion of immunized spleen cells and mouse myeloma cells (Fig. 3). The hybridoma cells that grew successfully in culture were cloned by limiting dilution methods (Fig. 4). Only the hybridoma clones that produced high titers of antibodies against rCatL, as screened by indirect ELISA, were selected. The antibody isotypes were determined by ELISA using the SBA Clonotyping™ System/HRP (SouthernBiotech, USA) as shown in Table 1.

1.3 Immunoblotting and cross reactivities studies

The immunobotting experiment revealed that all MoAbs reacted with a single band of rFgCatL which has a molecular weight (MW) of 30 kDa (Fig. 5). However, when tested against WB antigens in all developmental stages (metacercariae, NEJ, 1, 3, 5-week-old juveniles and adults), and TA and ES antigens from adult *F. gigantica*, these MoAbs reacted intensely with native CatL which appeared as a single band at MW 27 kDa in all WB and ES extracts. In contrast, no positive band was detected in adult TA fraction (Fig. 6A). When similar antigenic fractions were analyzed with PoAbs against native CatL1, the positive band was observed at MW 27 kDa which confirmed that the protein detected by MoAb was CatL1 (Fig. 6B). In addition, there was another positive band at MW 27 kDa in

adult TA, 17 kDa and 14 kDa in WB extracts of metacercariae, NEJ, 1, 3, 5-week-old juveniles, adult WB and adult ES (Fig. 6B).

For the cross-reactivity study, the SDS-PAGEs of WB antigens from *F. gigantica,* other trematode, cestode and nematode parasites were shown in Figure 7A. No positive band was detected when the MoAb reacted with WB antigens from five trematode parasites (*P. cervi, E. pancreaticum, G. explanatum*, *S. spindale* and *S. mansoni*), from two cestode parasites (*M. benedeni and A. centripunctata*), and from three nematode parasites (*Trichuris* sp., *H. placei* and *S. labiato-papillosa*), while the band was very conspicuous in adult *F. gigantica* WB antigen (Fig. 7B).

1.4 Immunolocalization of CatL

The distribution of CatL in each development stages of *F. gigantica* (metacercariae, NEJ, 1, 3, 5-week-old juveniles, adults) was examined by immunoperoxidase staining using the MoAbs as probes. All MoAbs exhibited similar immunoperoxidase staining pattern as represented by MoAb 4E3 (Fig. 8) which showed the strongest reaction. The positions and intensities of the brownish reaction products indicated the location and relative concentration of CatL that were bound to the MoAb. The myeloma culture fluid, used as a negative control, showed no brownish staining in any tissues of the parasite (Figs. 8A).

Consistent with the immuoblotting analysis, the positive immunostaining was detected in both caecal epithelium and in the lumen of the caecum of metacercariae, NEJ, 1, 3 and 5-week-old juveniles *F. gigantica*, while the tegument, tegumental cells, and parenchymal cells were not stained (Figs. 8B-F). Similar to juveniles, immunostaining of the adult parasite sections was intense in both the caecal epithelium and in lumen of the caecum (Fig. 8G and H), while the tegument, tegumental cells, vitelline cells, testes, muscle and parenchymal cells showed no staining (Fig. 8G).

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1.4 Production of the PoAb against rCatL antigens of *F. gigantica*

Antibody titers of rabbit serum against rCatL antigens of *F.gigantica* were tested by indirected ELISA technique. A rabbit showing OD value higher than mean value of the pre-immune serum plus 3 standard deviations (SD) is considered to be the high responder. The high responder was used for purification.

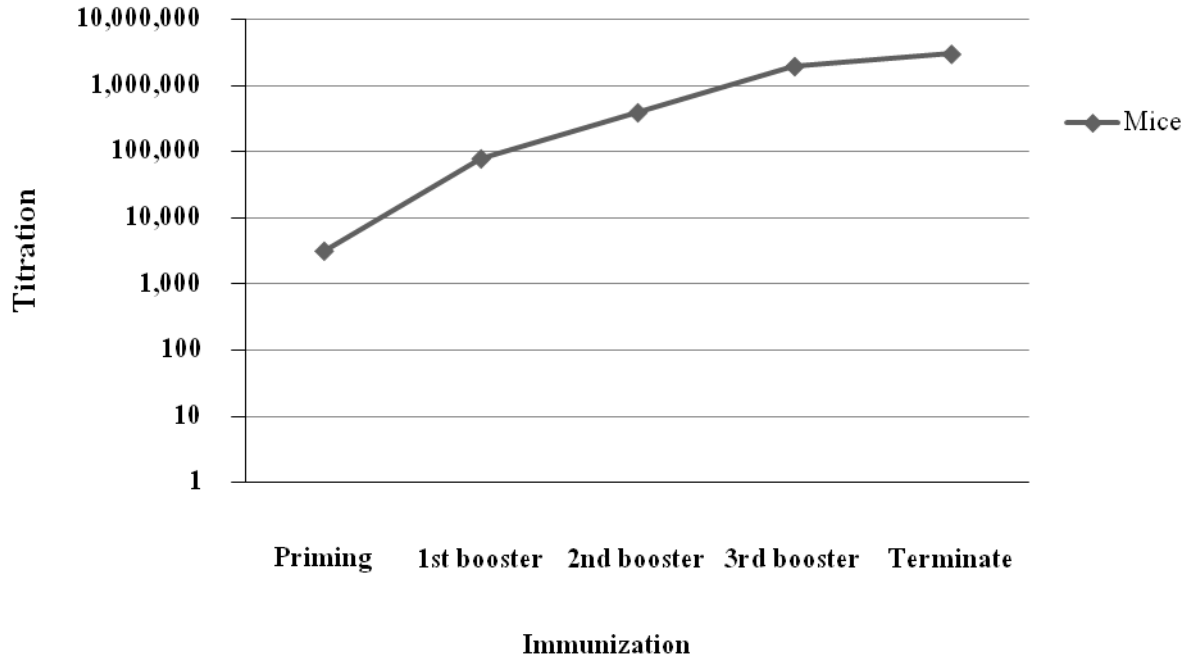


Figure 2 Indirected ELISA exhibited antibody titers of mouse serum against rCatL antigen of *F. gigantica*.

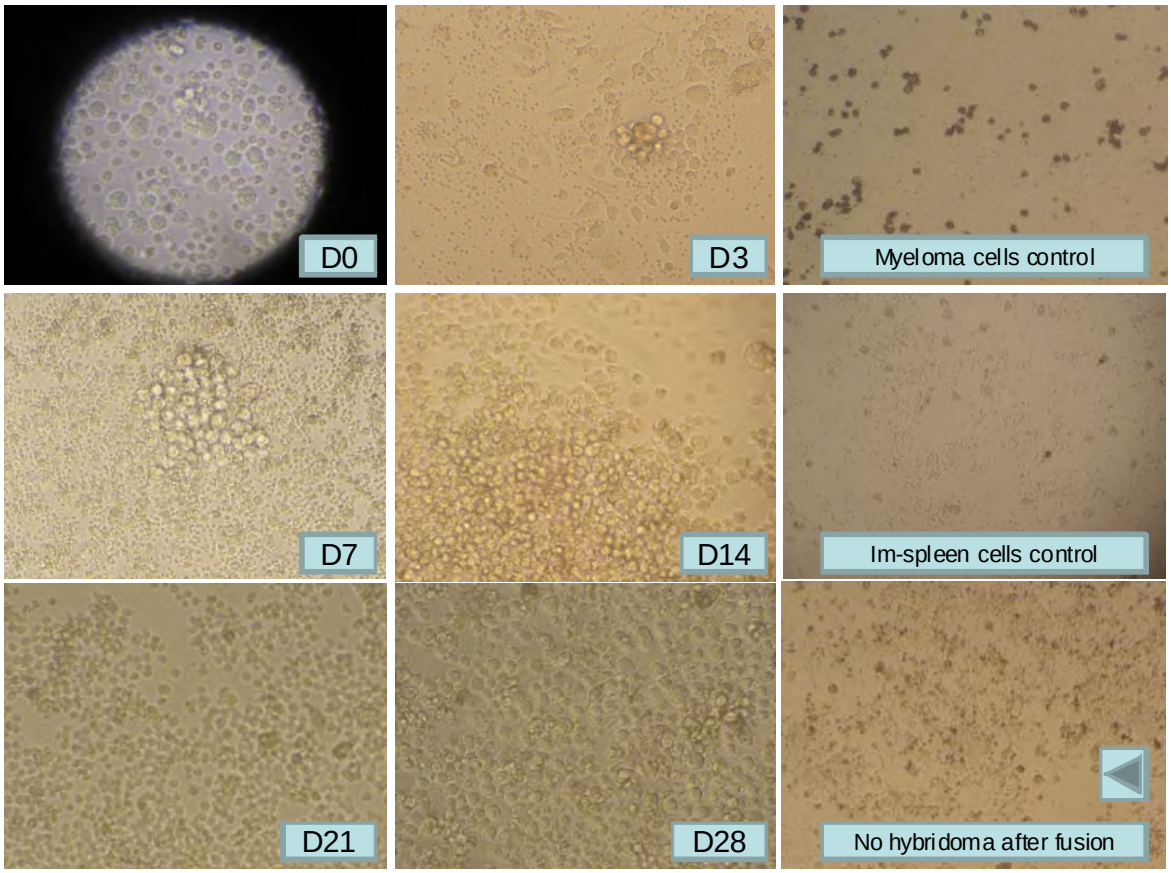


Figure 3 Fusion of spleen cells immunized with rCatL and mouse myeloma cells.

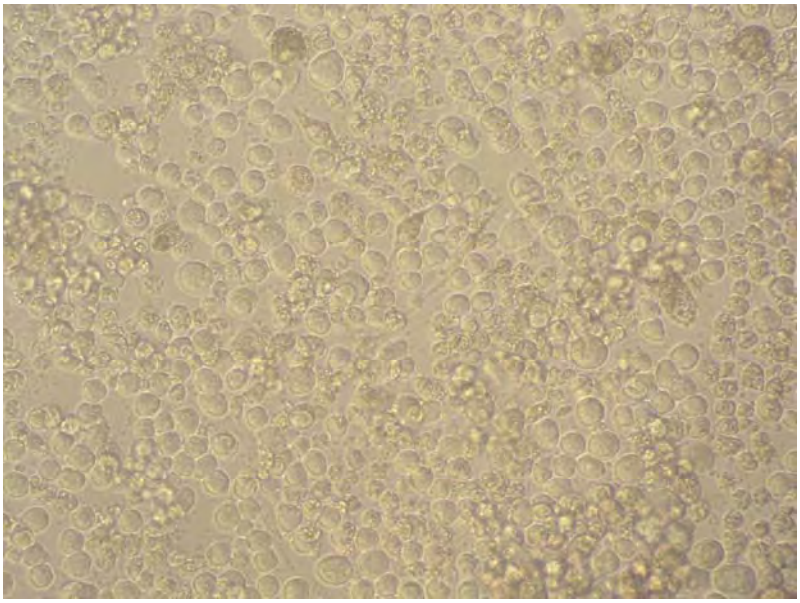


Figure 4 The hybridoma cells that grew successfully in culture media were cloned by limiting dilution technique.

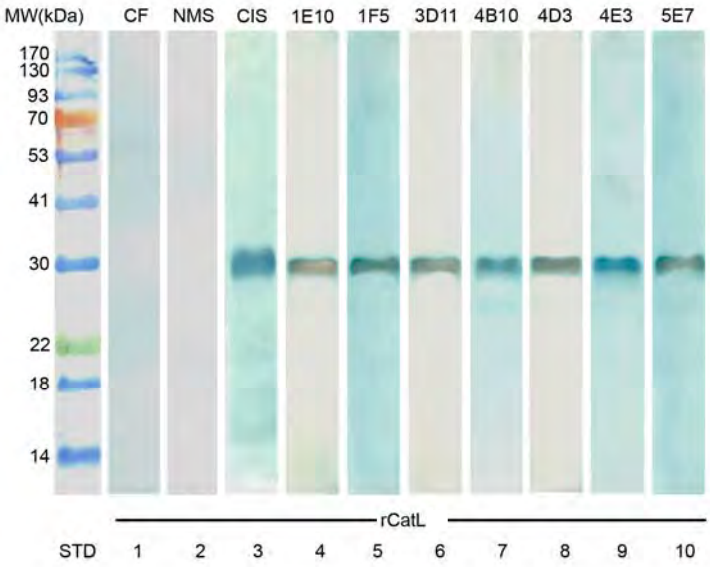
Table 1 Indirect ELISA result showed the OD values of monoclonal antibodies specific against rCatL.

Antibody isotyping			
Number	**Clone**	**Heavy chain (OD 405)**	**Light chain (OD 405)**
1	1E10-2D2	IgM	κ
2	1F5-3E3	IgM	κ
3	3D11-3B9	IgM	κ
4	3D11-3E9	IgM	κ
5	3D11-3F11	IgM	κ
6	4B10-8B4	IgG_1	κ
7	4B10-8C1	IgG_1	κ
8	4B10-8C3	IgG_1	κ
9	4B10-8D2	IgG_1	κ
10	4B10-8D3	IgG_1	κ
11	4B10-8E3	IgG_1	κ
12	4B10-8F2	IgG_1	κ
13	4E3-10E5	IgG_1	κ

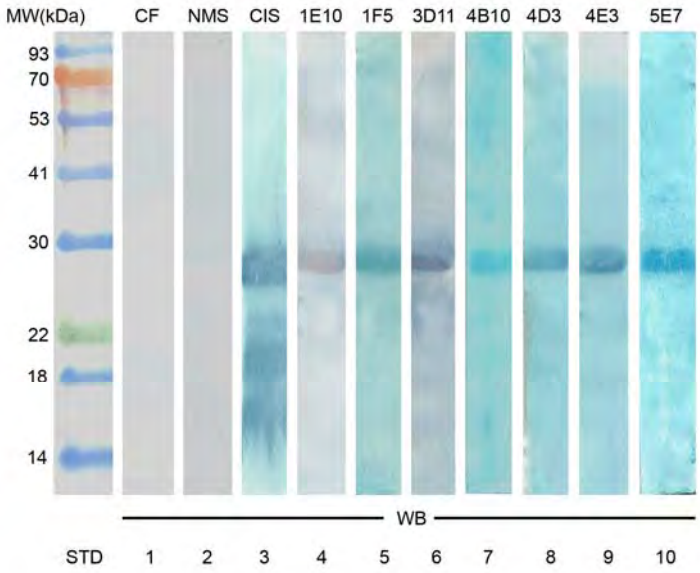
Figure 5 Determination of the immuno-reactivity of MoAbs against native and recombinant *F. gigantica* cathepsin L (rFgCatL).

(A) Immunoblot patterns of the rFgCatL reacted with myeloma culture fluid-CF (lane 1), normal mouse serum-NMS (lane 2), cattle infected serum-CIS (lane 3), MoAb 1E10 (lane 4), 1F5 (lane 5), 3D11 (lane 6), 4B10 (lane 7), 4D3 (lane 8), 4E3 (lane 9) and 5E7 (lane 10).

(B) Immunoblot patterns of *F. gigantica* whole body extract (WB) antigens reacted with myeloma culture fluid-CF (lane 1), normal mouse serum-NMS (lane 2), cattle infected serum-CIS (lane 3), MoAb 1E10 (lane 4), 1F5 (lane 5), 3D11 (lane 6), 4B10 (lane 7), 4D3 (lane 8), 4E3 (lane 9) and 5E7 (lane 10). STD is the lane containing standard protein molecular weight markers on the left side.


MW(kDa)
CF
NMS
CIS
1E10
1F5
3D11
4B10
4D3
4E3
5E7
170
130
93
70
53
41
30
22
18
14
rCatL
STD
1
2
3
4
5
6
7
8
9
10

A

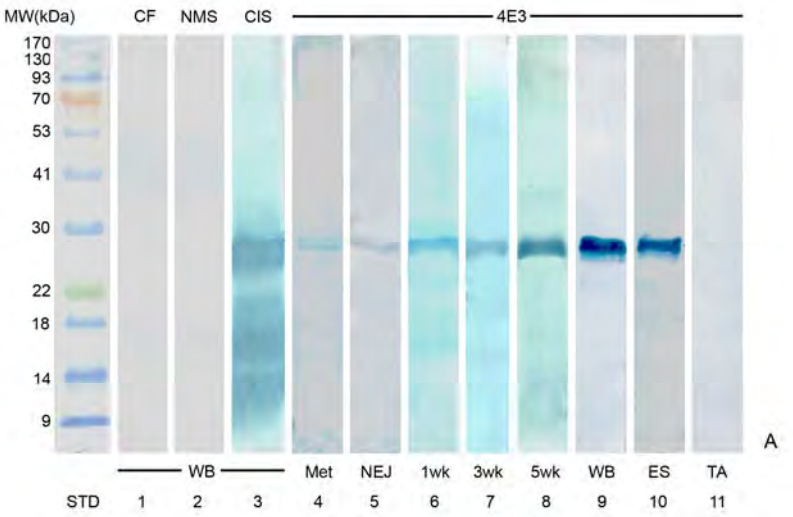

MW(kDa)
CF
NMS
CIS
1E10
1F5
3D11
4B10
4D3
4E3
5E7
93
70
53
41
30
22
18
14
WB
STD
1
2
3
4
5
6
7
8
9
10

B

Figure 6 Determination of the immuno-reactivity of MoAb clone 4E3 and PoAb against native CatL.

(A) Immunoblot analysis of *F. gigantica* whole body extract (WB) antigens reacted with myeloma CF (lane 1), NMS (lane 2), CIS (lane 3), while metacercariae (Met) in lane 4, NEJ in lane 5, 1-week-old juvenile (1wk) in lane 6, 3-week-old juvenile (3wk) in lane 7, 5-week-old juvenile (5wk) in lane 8, WB in lane 9, excretory-secretory (ES) in lane 10 and tegumental antigen (TA) antigens in lane 11 were blotted with MoAb 4E3. Other clones of MoAb showed similar pattern and were not shown.

(B) Immunoblot analysis of *F. gigantica* WB antigens blotted with myeloma CF (lane 1), NMS (lane 2), CIS (lane 3), while metacercariae (Met) in lane 4, NEJ in lane 5, 1-week-old juvenile (1wk) in lane 6, 3-week-old juvenile (3wk) in lane 7, 5-week-old juvenile (5wk) in lane 8, WB in lane 9, excretory-secretory (ES) in lane 10 and tegumental antigen (TA) antigens in lane 11 were blotted with polyclonal antibodies (PoAb) against native CatL. STD is the lane containing standard protein molecular weight markers.


MW(kDa)
CF
NMS
CIS
4E3
170
130
93
70
53
41
30
22
18
14
9
WB
Met
NEJ
1wk
3wk
5wk
WB
ES
TA
STD
1
2
3
4
5
6
7
8
9
10
11
A

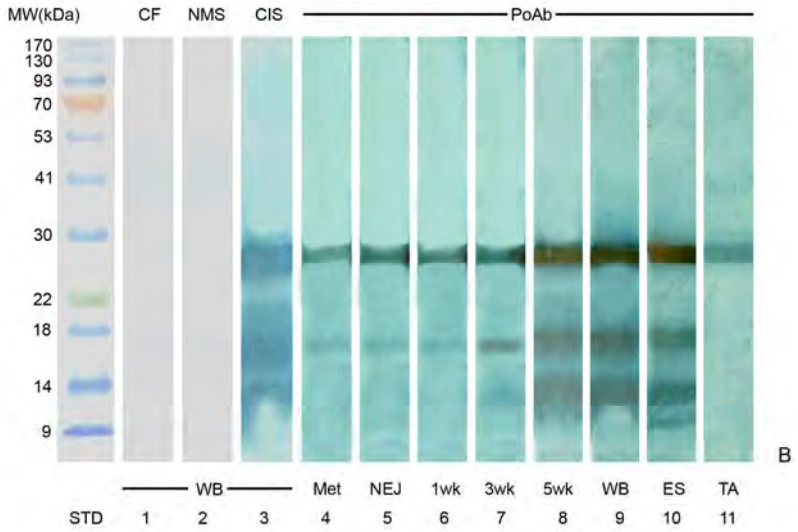

MW(kDa)
CF
NMS
CIS
PoAb
170
130
93
70
53
41
30
22
18
14
9
WB
Met
NEJ
1wk
3wk
5wk
WB
ES
TA
STD
1
2
3
4
5
6
7
8
9
10
11
B

Figure 7 SDS-PAGE of whole body (WB) antigens from *F. gigantica,* other trematode, cestode and nematode parasites showing the protein profiles after Coomassie blue staining, and immunoblot analysis of these proteins using MoAb clone 4E3 as probe.

(A) Coomassie blue staining of SDS-PAGE separated WB antigens from *F. gigantica,* other trematode, cestode and nematode parasites, showing protein bands with a wide range of molecular weights. WB = *F. gigantica* (lane1), Pc = *P. cervi* (lane 2), Ep = *E. pancreaticum* (lane 3),Ge = *G. explanatum* (lane 4), Ss = *S. spindale* (lane 5), Sm = *S. mansoni* (lane 6), Mb = *Moniezia benedeni* (lane 7), Ac = *Avitellina centripunctata* (lane 8), Ts = *Trichuris* sp. (lane 9), Hp = *Haemonchus placei* (lane 10) and Sp = *Setaria labiato-papillosa* (lane 11). STD is the lane containing standard protein molecular weight markers.

(B) Immunoblot analysis showing the cross-reactivity of MoAb clone 4E3 with WB antigens from *F. gigantica* (WB) (lane 4), Pc = *P. cervi* (lane 5), Ep = *E. pancreaticum* (lane 6),Ge = *G. explanatum* (lane 7), Ss = *S. spindale* (lane 8), Sm = *S. mansoni* (lane 9), Mb = *Moniezia benedeni* (lane 10), Ac = *Avitellina centripunctata* (lane 11), Ts = *Trichuris* sp. (lane 12), Hp = *Haemonchus placei* (lane 13) and Sp = *Setaria labiato-papillosa* (lane 14). The controls show WB antigens from *F. gigantica* adult blotted with myeloma CF (lane 1), NMS (lane 2) and CIS (lane 3). Other clones of MoAb showed similar pattern and were not shown. STD is the lane containing standard protein molecular weight markers.

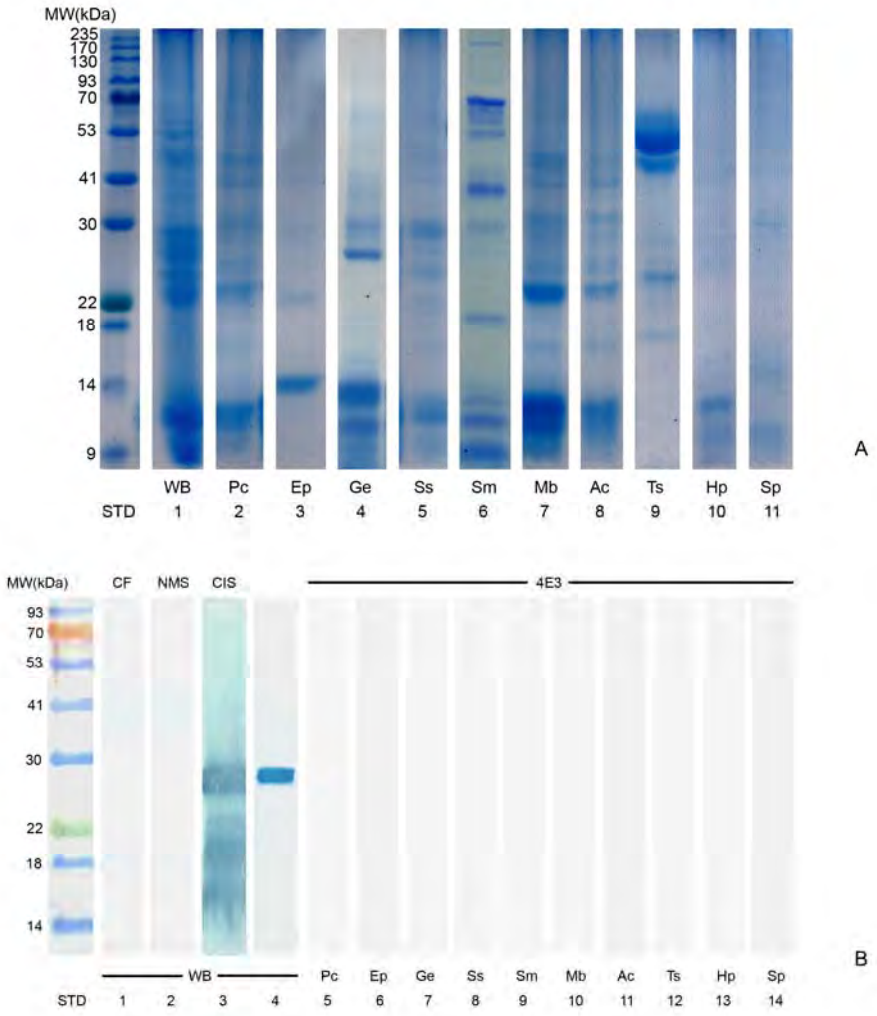

MW(kDa)
235
170
130
93
70
53
41
30
22
18
14
9
WB Pc Ep Ge Ss Sm Mb Ac Ts Hp Sp
STD 1 2 3 4 5 6 7 8 9 10 11
A
MW(kDa)
CF
NMS
CIS
4E3
93
70
53
41
30
22
18
14
WB
Pc Ep Ge Ss Sm Mb Ac Ts Hp Sp
STD 1 2 3 4 5 6 7 8 9 10 11 12 13 14
B

Figure 8 Light micrographs of paraffin sections of various stages of *F. gigantica* stained by immunoperoxidase technique using MoAb specific to rFgCatL as a probe.

(A) A negative control section of an adult parasite stained with myeloma culture fluid, showing unstained caecum (Ca), parenchymal cells (Pc), tegument (Te), spine (Sp), muscle (Mu) and vitelline cells (Vi).

(B) A high magnification micrograph of a metacercarial section showing intense staining in the caecal epithelium and in the lumen of the caecum (Ca), while tegument (Te), parenchymal cells (Pc), outer cyst wall (Cw1) and inner cyst wall (Cw2) were not stained.

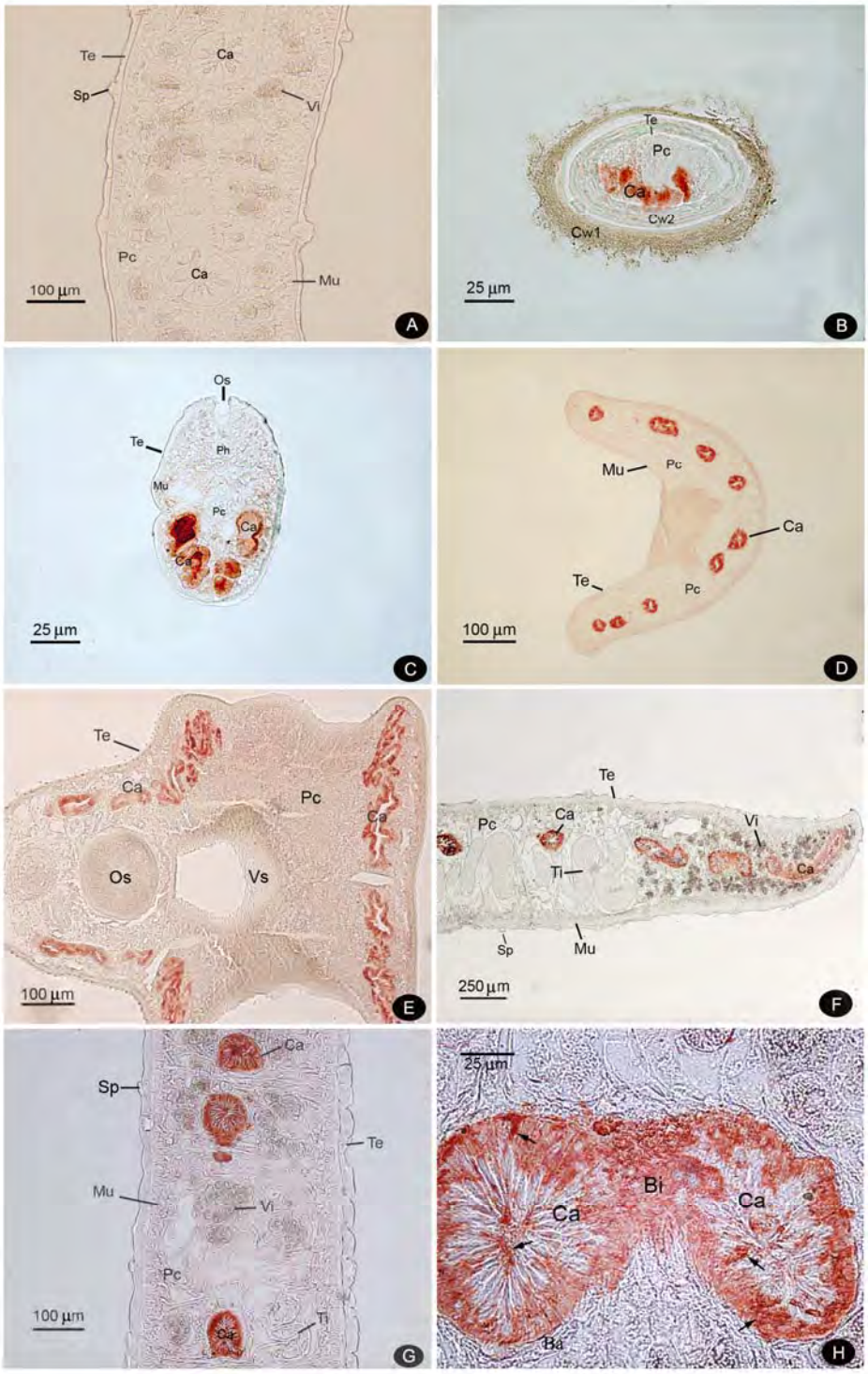
(C) A high magnification micrograph of a NEJ showing intense staining in the caecal epithelium and in the lumen of the caecum (Ca), while no staining was detected in oral sucker (Os), pharynx (Ph), tegument (Te), muscle (Mu), and parenchymal cells (Pc).

(D) A high magnification micrograph of a 3-week-old juvenile showing intense staining in the caecal epithelium and in the lumen of the caecum (Ca), while the tegument (Te), parenchymal cells (Pc) and muscle (Mu) were not stained.

(E) A medium magnification micrograph of a 5-week-old juvenile showing intense staining in the caecal epithelium and in the lumen of the caecum (Ca), while the oral sucker (Os), ventral sucker (Vs), tegument (Te), muscle (Mu) and parenchymal cells (Pc) were not stained.

(F-G) Low and medium magnification micrographs of adult *F. gigantica* sections showing intense staining only in the caecal epithelium and in the lumen of the caecum (Ca), while no staining was observed in the tegument (Te), spine (Sp), muscle (Mu), parenchymal cells (Pc), testes (Ti) and vitelline cells (Vi).

(H) A higher magnification micrograph of an adult *F. gigantica* section showing strong intense staining the cytoplasm of bifurcation (Bi) of caecal epithelium (Ca) and accumulation of the granules (arrows) in the apical and basal cytoplasm, while the basal lamina (Ba) was not stained.


Te
Ca
Sp
Vi
Pc
Ca
Mu
100 µm
A
Te
Pc
Ca
Cw2
Cw1
25 µm
B
Os
Te
Ph
Mu
Pc
Ca
Ca
25 µm
C
Mu
Pc
Ca
Te
Pc
100 µm
D
Te
Ca
Pc
Ca
Os
Vs
100 µm
E
Te
Pc
Ca
Vi
Ti
Ca
Sp
Mu
250 µm
F
Ca
Sp
Te
Mu
Vi
Pc
100 µm
Ti
Ca
G
25 µm
Bi
Ca
Ca
Ba
H

2. Purification of the MoAbs and PoAbs against rCatL of *F. gigantica*

2.1 Purification of the MoAbs

Mouse MoAbs was fractioned by ammonium sulfate precipitation followed by gel filtration chromatography on Sephadex G-200 as described in section of materials and methods. The chromatographic profiles and the ELISA antibody activity are shown in Figure 9, 10, 12, 13, 15, 16 and Table 2-4. Various preparations of MoAbs obtained from the fraction were subjected to SDS-PAGE in 12.5% separating gel and 4% stacking gel (Fig. 11, 14 and 17). The result showed the structure of immunoglobulin (MW 5,500 Da of heavy chain and 2,200 Da of light chain). Fractions containing anti-rCatL activity were pooled and concentrated by ultrafiltration using Diaflo PM-10 membrane.

2.2 Purification of the PoAbs

Rabbit immunoglobulin was fractioned by ammonium sulfate precipitation followed by ion-exchange chromatography on DEAE cellulose as described in materials and methods. The chromatographic profiles and the ELISA antibody activity are shown in Figure 18 and 19. Various preparations of rabbit immunoglobulin obtained from the fraction were subjected to SDS-PAGE in 12.5% separating gel and 4% stacking gel (Fig. 20). The result showed the structure of IgG immunoglobulin (MW 5,500 Da of heavy chain and 2,200 Da of light chain). Fractions containing anti-rCatL activity were pooled and concentrated by ultrafiltration using Diaflo PM-10 membrane. The specificity of rabbit anti-rCatL antibody was studied by immunoblotting using various trematode and nematode parasite antigens. After purification, the immunoblot detection showed that this rabbit antibody was very specific in the detection of rCatL and did not cross react with other worm antigens.

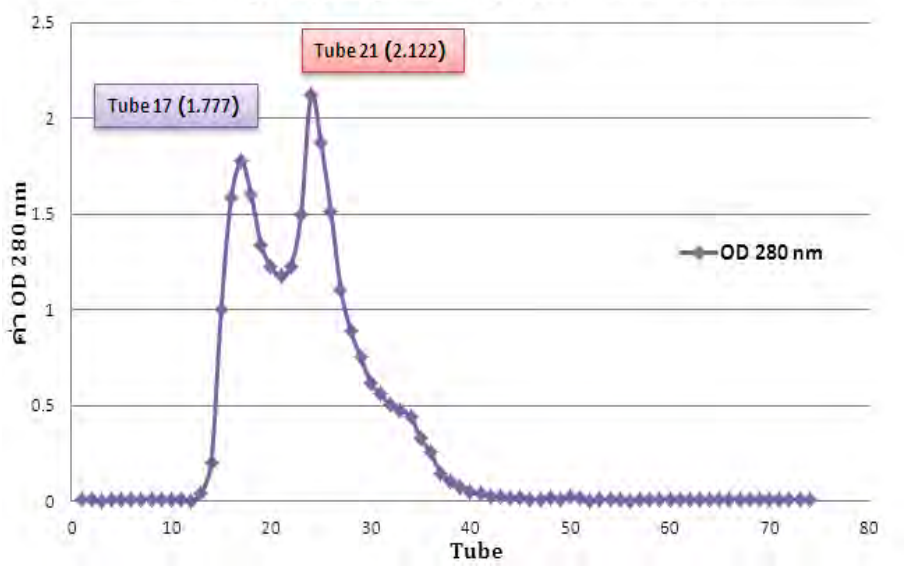


Figure 9 The chromatographic antibody profiles of mouse monoclonal antibody (IgM 1F5-3E3) against rCatL on a Sephadex G-200 column.

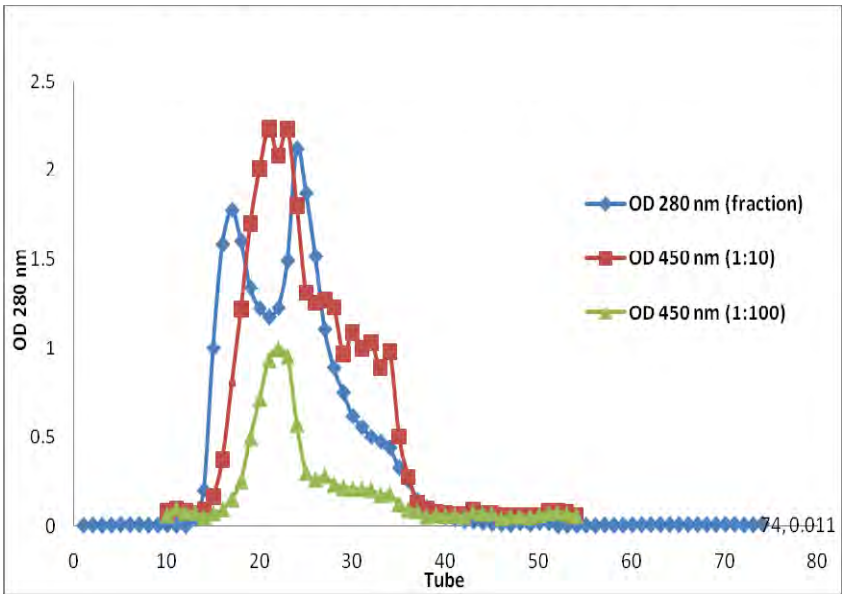


Figure 10 Activity of MoAb (IgM 1F5-3E3) specific against rCatL of *F. gigantica*.

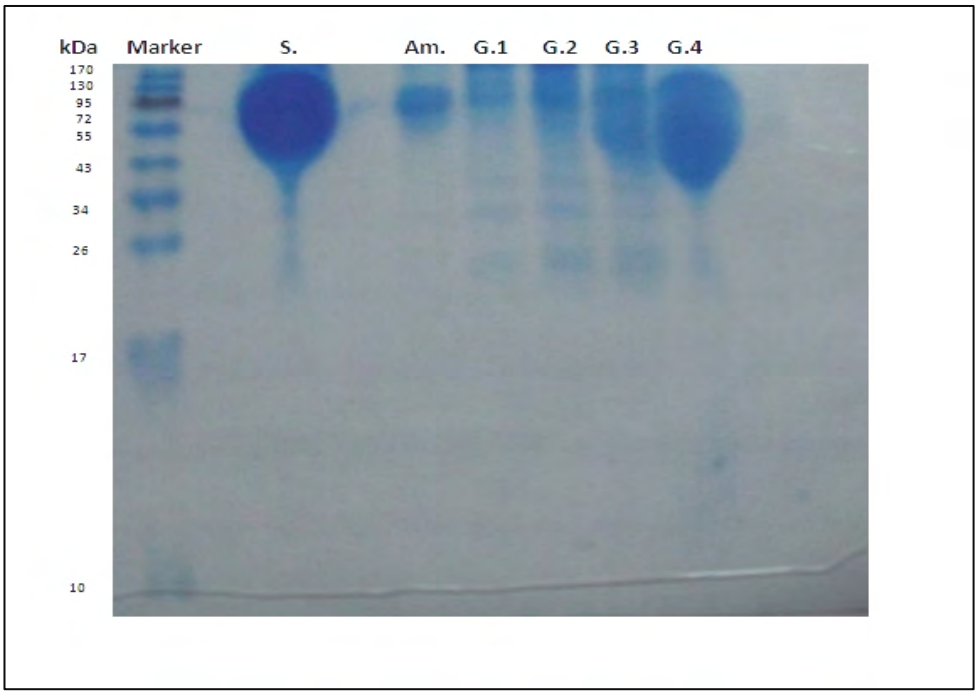


Figure 11 SDS-PAGE of various preparations of mouse monoclonal antibody (IgM 1F5-3E3) against rCatL.

Marker: Pre-stained marker.

S: Sample of hybridoma IgM.

Am: Ammonium sulfate precipitated IgM.

G1-G4: Pooled fractions of Sephadex G-200 purified IgM.

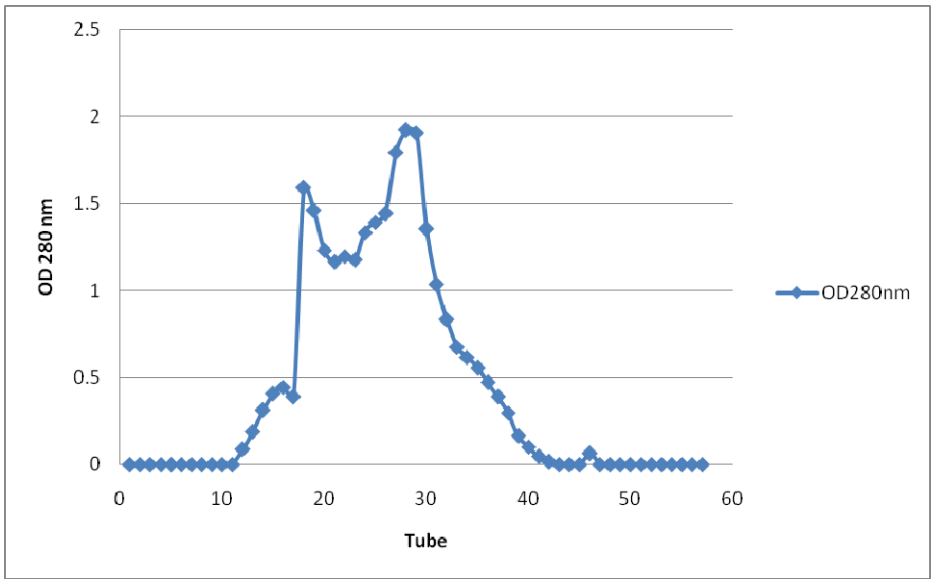


Figure 12 The chromatographic antibody profiles of mouse monoclonal antibody (IgG_1 4E3-10E5) against rCatL on a Sephadex G-200 column.

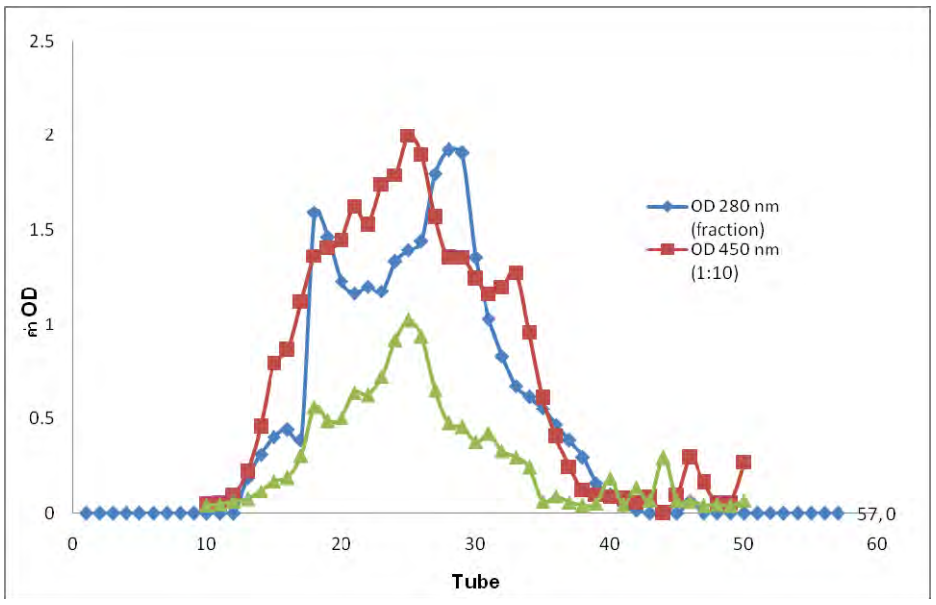


Figure 13 Activity of MoAb (IgG_1 4E3-10E5) specific against rCatL of *F. gigantica*.

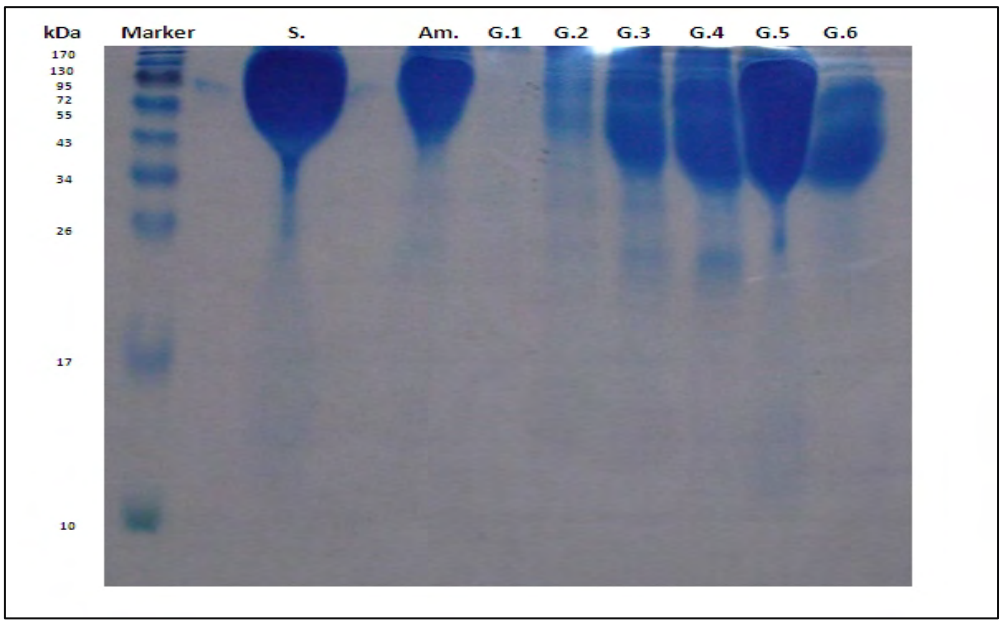


Figure 14 SDS-PAGE of various preparations of mouse monoclonal antibody (IgG_1 4E3-10E5) against rCatL.

Marker: Pre-stained marker.

S: Sample of hybridoma IgG_1.

Am: Ammonium sulfate precipitated IgG_1.

G1-G6: Pooled fractions of Sephadex G-200 purified IgG_1.

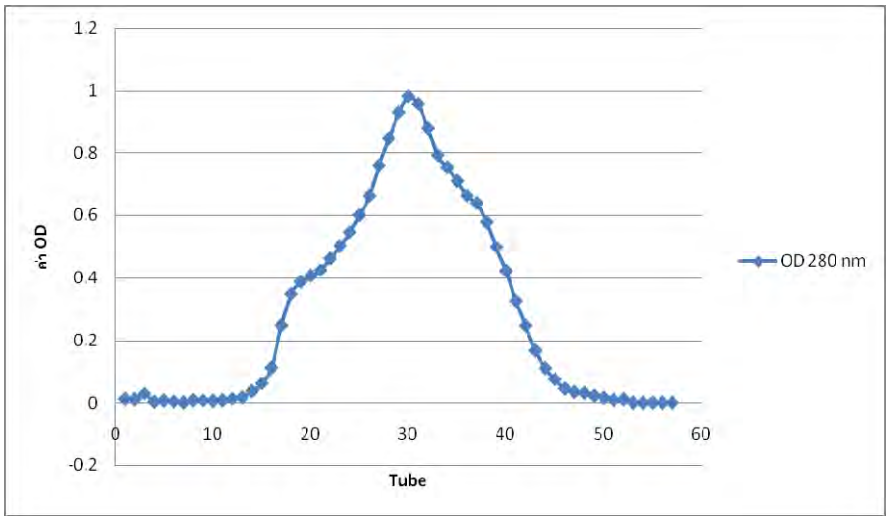


Figure 15 The chromatographic antibody profiles of mouse monoclonal antibody (IgG_1 4B10-8D3) against rCatL on a Sephadex G-200 column.

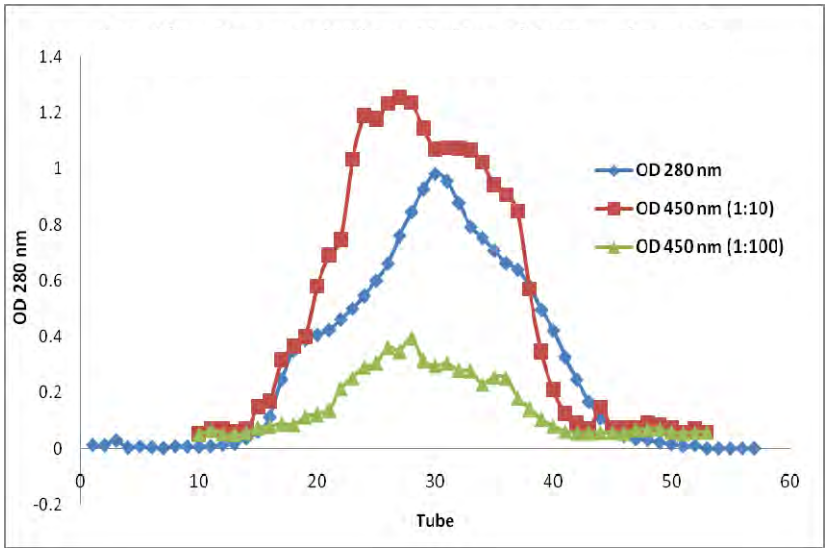


Figure 16 Activity of MoAb (IgG_1 4B10-8D3) specific against rCatL of *F. gigantica*.

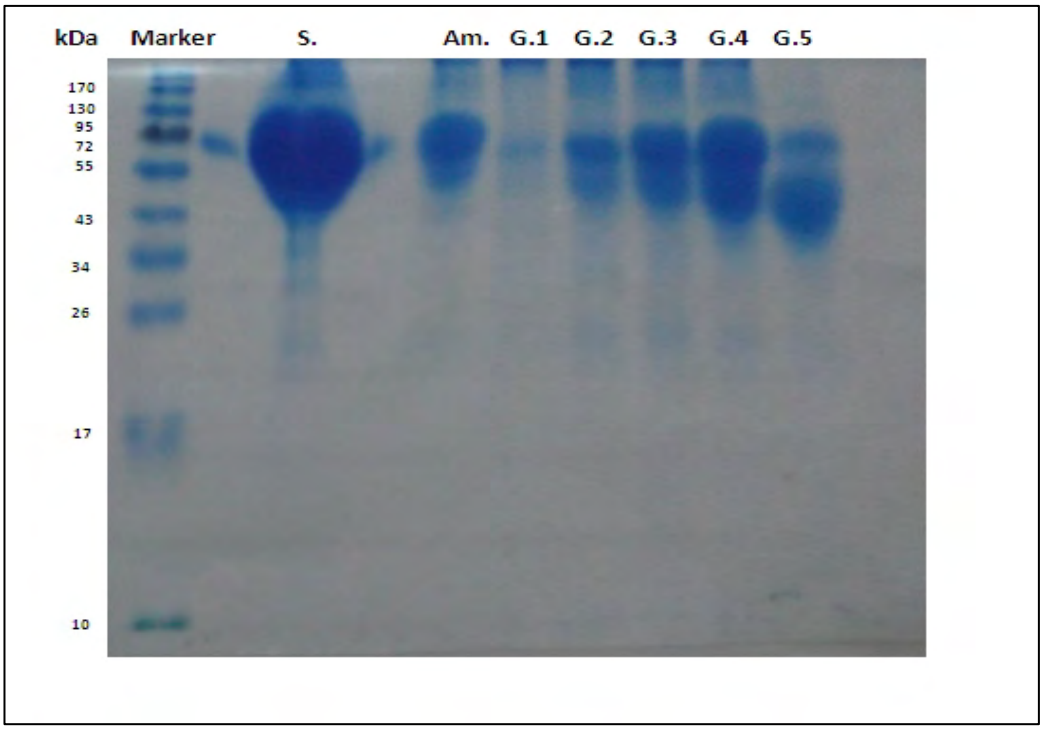


Figure 17 SDS-PAGE of various preparations of mouse monoclonal antibody (IgG_1 4B10-8D3) against rCatL.

Marker: Pre-stained marker.

S: Sample of hybridoma IgG_1.

Am: Ammonium sulfate precipitated IgG_1.

G1-G5: Pooled fractions of Sephadex G-200 purified IgG_1.

Table 2 Indirect ELISA results showed activity of MoAb (IgM, 1F5-3E3) specific against rCatL of *F. gigantica* after purification. Note: G.1 = Group 1; G.2 = Group 2; G.3 = Group 3; G.4 = Group 4.

Group	OD 280 nm (Average)	OD 450 nm (1:10)	OD 450 nm (1:100)
G.1	1	0.189	0.166
G.2	1.655	0.337	0.173
G.3	1.292	1.354	0.472
G.4	2.122	1.112	0.351

Table 3 Indirect ELISA results showed activity of MoAb (IgG_1, 4E3-10E5) specific against rCatL of *F. gigantica* after purification. Note: G.1 = Group 1; G.2 = Group 2; G.3 = Group 3; G.4 = Group 4; G.5 = Group 5; G.6 = Group 6.

Group	OD 280 nm (Average)	OD 450 nm (1:10)	OD 450 nm (1:100)
G.1	0.188	0.271	0.083
G.2	0.766	1.439	0.471
G.3	1.197	1.662	1.033
G.4	1.335	1.808	1.376
G.5	1.874	1.673	0.973
G.6	0.74	1.441	0.615

Table 4 Indirect ELISA results showed activity of MoAb (IgG_1, 4B10-8D3) specific against rCatL of *F. gigantica* after purification. Note: G.1 = Group 1; G.2 = Group 2; G.3 = Group 3; G.4 = Group 4; G.5 = Group 5.

Group	OD 280 nm (Average)	OD 450 nm (1:10)	OD 450 nm (1:100)
G.1	0.32	0.309	0.093
G.2	0.53	0.702	0.247
G.3	0.756	1.101	0.339
G.4	0.88	0.934	0.282
G.5	0.62	0.646	0.146

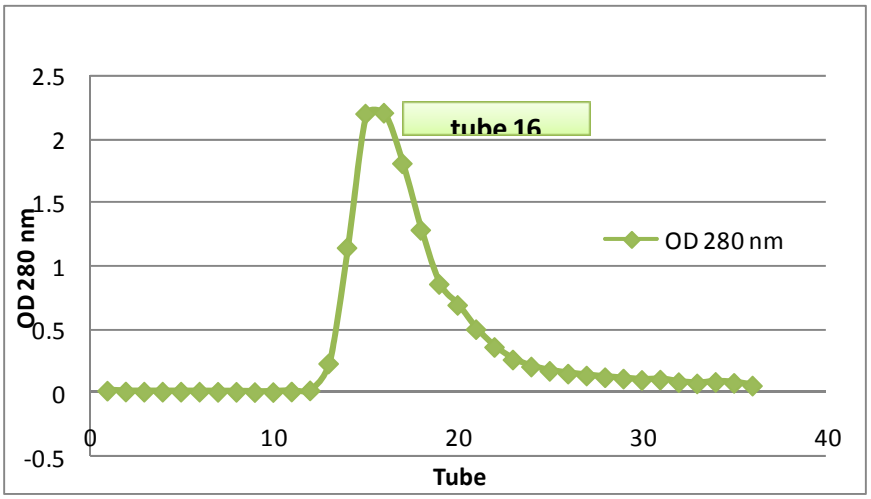


Figure 18 The chromatographic antibody profiles of rabbit polyclonal antibody against rCatL on a DEAE column.

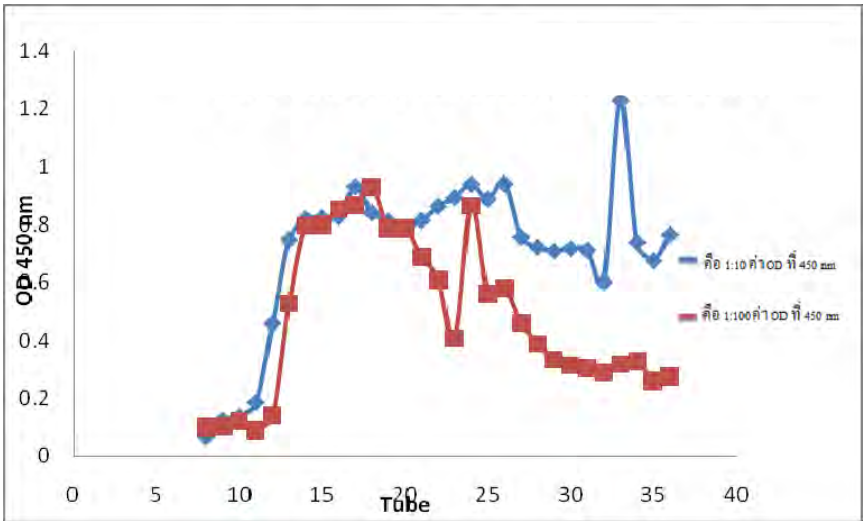


Figure 19 Activity of rabbit polyclonal antibody specific against rCatL of *F. gigantica*.

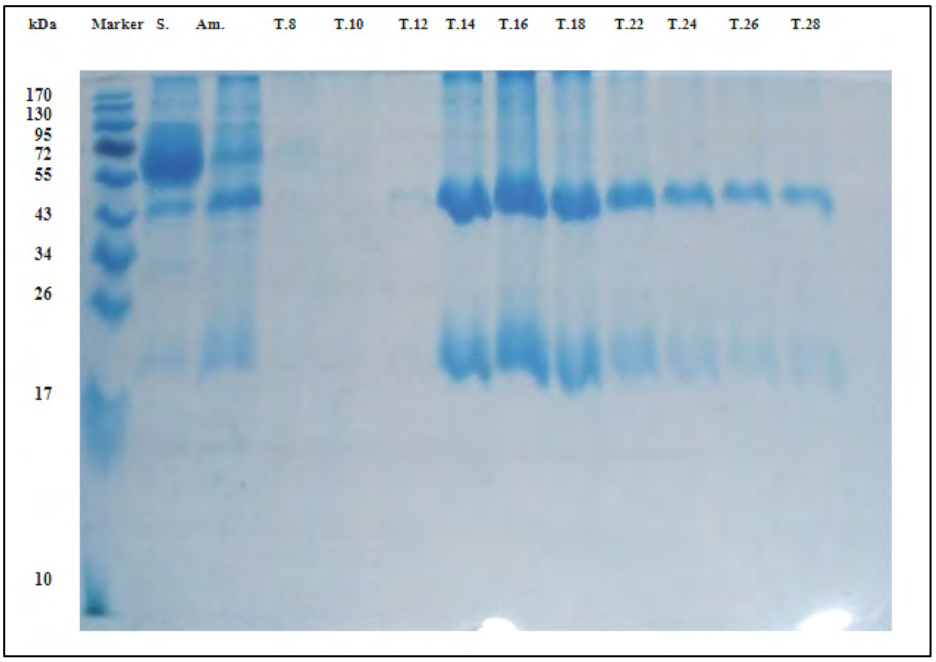


Figure 20 SDS-PAGE of various preparations of rabbit monoclonal antibody against rCatL.

Marker: Pre-stained marker.

S: Sample of rabbit serum.

Am: Ammonium sulfate precipitated immunoglobulin.

T8-T28: fractions of DEAE purified immunoglobulin.

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3. Development of a monoclonal antibody (MoAb)-based sandwich enzyme-linked immunosorbent assay (sandwich ELISA) for the detection of circulating CatL in serum of experimentally and naturally infected animals with fasciolosis caused by *F. gigantica*

3.1 Sensitivity and specificity of the sandwich ELISA

The specificity of sandwich ELISA and the lower detectionlimitThe ELISA cut-off value was calculated as the mean opticaldensity (OD) of control non-infected sera plus 3 standard devia-tions (SD), which was at 0.116. The OD readings equal to or lessthan the cut-off value were considered negative while those readings greater than the cut-off value were considered positive. The lower detection limit (sensitivity) of this assay system was determined using different concentrations of rCatL and WB, ES, TA of *F. gigantica*. Based on the lowest concentrations of antigen that still gave positive OD values, this assay could detect rCatL and CatL in WB, ES, TA of *F. gigantica* at the lowest concentrations of 3, 30 and 40 pg/ml, respectively (Fig. 21). Likewise, the sandwich ELISA described herein was highly specific for CatL, as no cross-reactivity was demonstrated when the assay was tested with various concentrations of other parasite antigens (Fig. 22).

3.2 Sandwich ELISA for the detection of circulating CatL antgen of *F. gigantica*

3.2.1 Experimentally infected mice

At day 35 post infection, the morbidity rate of infected mice was 100% and no mortality has occurred throughout this study period. The fluke recoveries varied from 1 to 18 flukes per mouse with an average of 8.67 + 5.52. For hepatic pathology, the migratory tracks were observed and appeared as irregular lines that scattered throughout the center of the hepatic lobes. In addition, the hepatic surfaces were covered with fibrinopurulent exudates that later caused adhesion of all of the liver lobes with adjacent organs inthe abdominal cavity. The total numbers of sera from mice infected with *F. gigantica* metacercariae examined and the numbers as well as the percentages of positive sera at day 1 to 35 post infection are shown in Table 5. The mean OD for antigen detection was significantly different from those of control sera at day 1, 4, 7 ($p < 0.05$), 21 and 35 ($p < 0.01$) post infection. The levels of detectable antigen in infected sera peaked at week 5^{th} post infection (Fig. 23). Sixty sera from mice infected with fasciolosis, 20 sera from mice infected with schistosomiasis, 20 sera from hamsters infected with opisthorchiasis, as well as 60 and 40 sera from non-infected mice and hamsters were

tested. The results shown in Fig. 24 revealed that 57 of 60 (95%) fasciolosis sera were positive, while all 140 (100%) sera from those with other infections and non-infected animals were negative. The ELISA cut-off value was at 0.116. Hence, this assay exhibited a sensitivity and specificity of 95% and 100%, respectively, with a positive predictive value of 100%, a negative predictive value of 97.9%, false positive rate of 0%, false negative rate of 5.3% and an accuracy of 98.5% (Table 6).

3.2.2 Naturally infected cattle and buffaloes

A total of 135 serum samples together with corresponding fecal samples from cattle were collected and examined. Sixty sera from cattle infected with fasciolosis, 50 from cattle infected with paramphistomosis, 10 from cattle infected with strongylid infections, 10 from cattle infected with trichuriasis, 5 from cattle infected with strongyloidiasis and 60 from non-infected cattle were tested. The results showed that 58 of 60 fasciolosis sera were positive, while all 75 sera from those infected with other parasites, and all 60 sera from normal controls were negative (Fig. 25). The ELISA cut-off value was at 0.118. Therefore, the method exhibited a sensitivity and specificity of 96.7% and 100%, respectively, with a positive predictive value of 100%, a negative predictive value of 98.5%, a false positive rate of 0%, a false negative rate of 3.4%, and an accuracy of 98.9% (Table 6).

3.3 Indirect ELISA for the detection of antibody against FABP and CatB3 of *F. gigantica*

The numbers of serum samples from infected mice being tested and percentages of positive detection during the period of 1–35 days post infection are shown in Table 5. From day 1 to 21, the percents of positive antibody detection were low, and the percent reached highest level (75%) at day 35 (Table 5). The mean OD for mouse antibody diagnosed fasciolosis at 35 days post infection is significantly different from that of the control sera OD ($p < 0.05$).

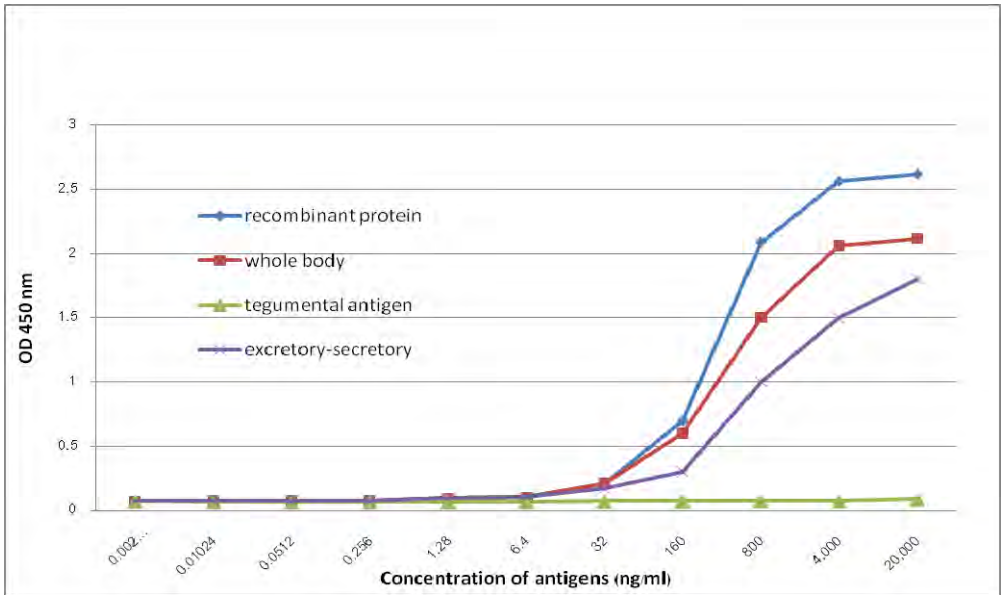

Figure 21 Sensitivity of known quantities of *F. gigantica* antigens. Titration of recombinant CatL (rCatL), whole boby (WB), excretory-secretory (ES) and tegumental (TA) antigens was analysed by sandwich ELISA.

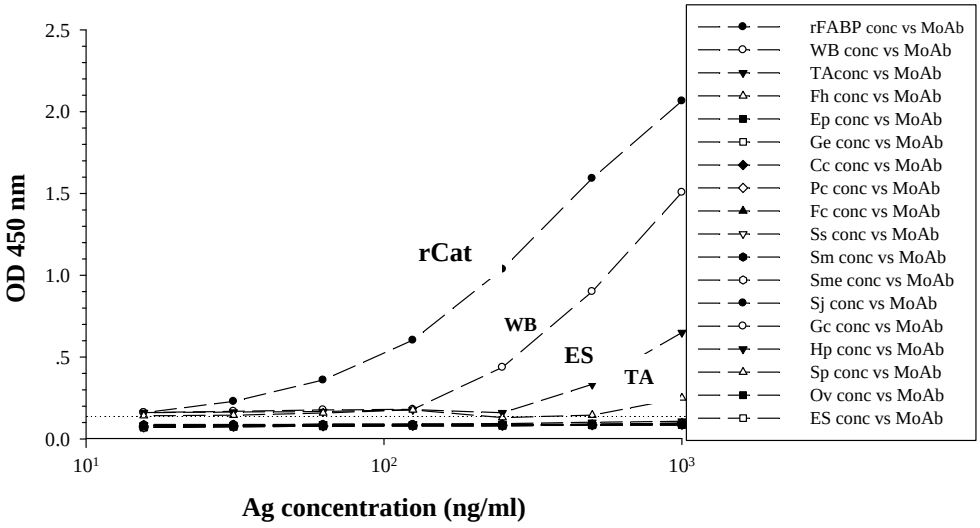

Figure 22 Cross-reactivities study of a MoAb-based sandwich ELISA to crude preparations of various trematode and nematode parasites. rCatL = recombinant CatL, WB = Whole body antigen, ES = Excretory-secretory antigen, TA = Tegumental antigen of *F. gigantica*, Fh = *F. hepatica*, Ep = *E. pancreaticum*, Ge = *G. explanatum*, Cc = *C. cotylophorum*, Pc = *P. cervi*, Fc = *F. cobboldi*, Ss = *S. spindale*, Sm = *S. mansoni*, Sj = *S. japonicum*, Sp = *S. labiato-papillosa,* Gc = *G. crumenifer*, Hp = *H. placei,* Sme = *S. mekongi*, Ov = *O. viverrini*.

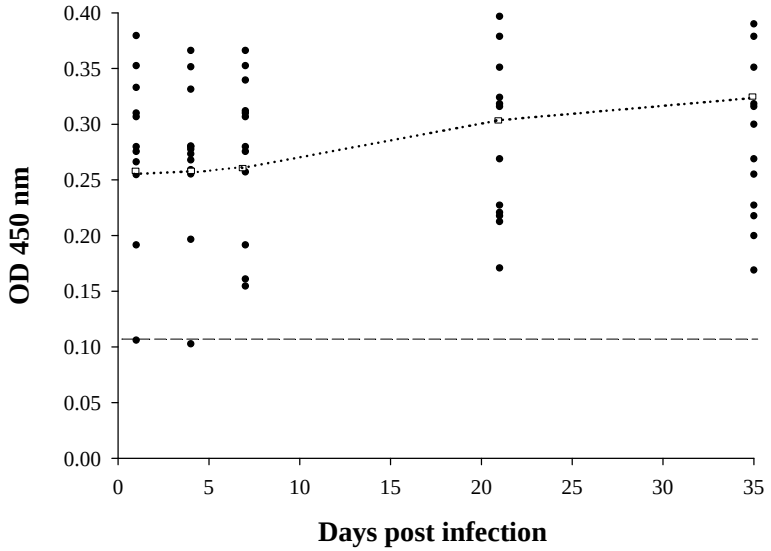

Figure 23 Detection of circulating CatL antigens of *F. gigantica* in the sera of the infected mice as compared to non-infected mice by sandwich ELISA. Black circles represent OD values of individual mouse serum; squares indicate mean OD values of all mice in the experimental group. The horizontal dashed line represents the cut-off level for a positive detection.

Table 5 Detection of circulating CatL antigen using sandwich ELISA compared to the detection of antibody against CatL in the sera of mice infected with *F. gigantica* using indirect ELISA. %P = percentage of positive sera; P = number of positive sera; N = number of sera tested for each day post infection.

	Sandwich ELISA		**Antibody detection ELISA**	
Days post infection	**%P**	**P/N**	**%P**	**P/N**
1	**83.33**	**11/12**	**8.33**	**1 /12**
4	**83.33**	**11/12**	**16.67**	**2 /12**
7	**83.33**	**11/12**	**25**	**3 /12**
21	**100**	**12/12**	**41.67**	**5 /12**
35	**100**	**12/12**	**75**	**9 /12**

Table 6 Calculation of diagnostic values of the sandwich ELISA for CatL antigen detection in sera of mice experimentally and cattle naturally infected with *F. gigantica*.

Calculations	Experimental infection (%)	Natural infection (%)
1. Sensitivity	95	96.7
2. Specificity	100	100
3. Positive predictive value	100	100
4. Negative predictive value	97.9	98.5
5. False positive rate	0	0
6. False negative rate	5.3	3.4
7. Accuracy	98.5	98.9

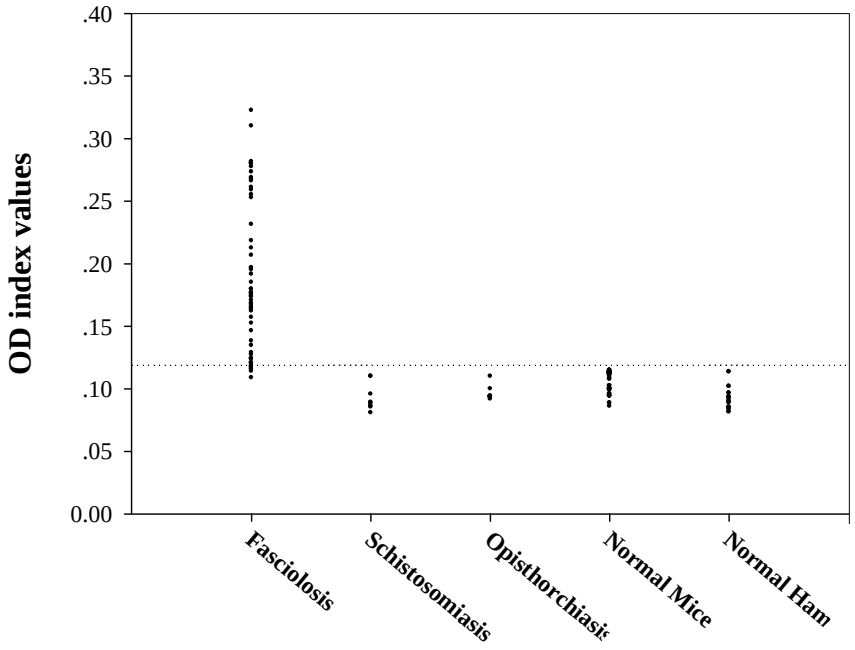

Figure 24 Scattergram showing the circulating CatL of *F. gigantica*. OD index values of serum samples obtained from mice infected with *F. gigantica* and *S. mansoni* as well as hamster infected with *O. viverrini* as measured by a MoAb-based sandwich ELISA. Sera from uninfected animals were used as test controls.

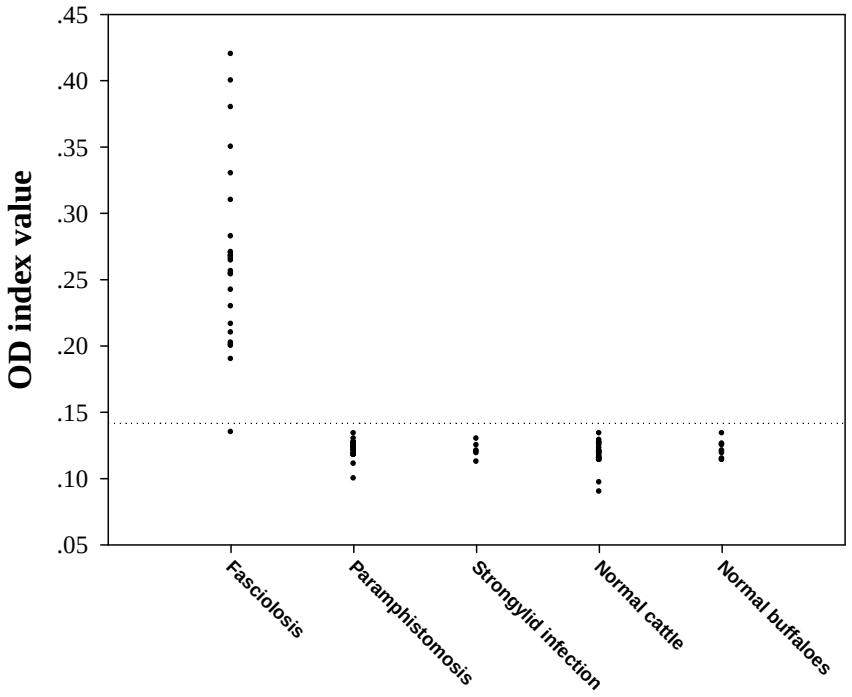

Figure 25 Scattergram showing the relative levels of circulating CatL antigens (OD values) in sera from cattle naturally infected with fasciolosis, paramphistomosis, Strongylid infection as measured by sandwich ELISA. Serum samples from non-infected cattle were used as controls.The horizontal dotted line represents the cut-off level for a positive detection.

CHAPTER VI
DISCUSSION

1. Production of MoAbs and PoAbs against rCatL antigens of *F. gigantica*

Several *F. hepatica* cathepsin L genes have been identified and isolated (Yamasaki and Aoki, 1993; Heussler and Dobbelaere, 1994). A recombinant cathepsin L1 of *F. hepatica* (rFhCL1) was also generated by expression of a cDNA from the adult stage (Roche et al., 1997). In this report, the recombinant protein of *F. gigantica* CatL1 (rFgCatL1) could be expressed in *Escherichia coli* BL21 (DE) and exhibited at a MW 30 kDa. The size of this recombinant protein was larger than the protein analyzed from the coding sequence because of the addition of hexahistidine tag at the C-terminus.

In this study, I could also produce MoAbs reacting with rFgCatL1. These MoAbs were both IgM and IgG_1–isotypes with κ light chain. Immunoblotting assay showed that these MoAbs could react with the rFgCatL1 at MW 30 kDa. Futhermore, they could also detect a single band of 27 kDa native protein in whole body extracts of *F. gigantica* metacercariae, NEJ, 1, 3, 5-week-old juveniles, adult WB and adult ES, but not in adult TA fraction. The MW of 27 kDa of this band is equal to MW of the putative protein deduced from the amino acid sequence of FgCatL1 (GenBank accession number AF112566) (Grams et al., 2001). However, it was recently reported that *F. gigantica* metacercariae and NEJ expressed catthepsin L1H (FgCatL1H) (GenBank accession number AY428949) in caecal epithelial cells instead of the CatL1 which is the adult isotype. Similarly, in *F. hepatica*, CatL1 was not expressed in metacercariae and NEJ (Robinson et al., 2008). The deduced amino acid sequence of the FgCatL1H shared 78.5% identity with FgCatL1 (Sansri et al., 2013). Thus the MoAbs might cross-react with the common epitope present in the juvenile-type CatL1H present in metacercariae and NEJ stage. This was reflected by the fact that these MoAbs exhibited less intensity upon binding with the antigens in the whole body extracts of *F. gigantica* metacercariae, NEJ, juveniles, than with the adult WB and ES, which could be due to binding affinity. Recently, we have produced a MoAb against FgCatL1H and found that it could cross reacted with FgCatL1 (unpublished result). In contrast to the MoAbs, PoAb could detect three bands of FgCatL1 at MW 27, 17 and 14 kDa in WB extracts of metacercariae, NEJ, 1, 3, 5-week-old juveniles, adult WB and ES which might reflect the existence of more than one isotype of these proteins in the *F. gigantica* which carried different epitopes. A phylogenetic analysis of the *Fasciola* Cat L family demonstrated that FgCatL1 belonged to a distinct

group, clade CL1C, as reported by Morphew et al. (2011). In addition, it has been shown that there are 14 different isoforms of *F. hepatica* Cat L proteases in the adult CL1, CL2 and CL5 Cat L protease clades (Morphew et al., 2011). The deduced amino acid sequence of the FgCatL1 shared 67.1–94.4% identity with other *F. hepatica* CatLs (Sansri et al., 2013). Hence, eventhough the monoclonal antibody 4E3 did not cross reacted with antigens from other trematode and nematode parasites, it could still cross-reacted with a possible common epitope in CatLs of *F. hepatica*, which needed to be tested in the future.

The localization and distribution studies revealed that our MoAbs could detect the native CatL1 or its homologs in epithelium and lumen of the caecum in metacercariae, NEJ, 1, 3 and 5-week-old juveniles and adult *F. gigantica* with a similar pattern to that described by Meemon et al. (2010) using a mouse polyclonal anti-serum against native cathepsin L of adult *F. gigantica*. This finding suggested that the native cathepsin L1 could be synthesized and stored in secretory vesicles of the gastrodermal epithelial cells in an inactive proenzyme form or procathepsin L in both juvenile and adult stage. Procathepsin L might then be secreted into an acidic environment of the caecal lumen where it was activated to become an active enzyme for the nutrient digestion. In *F. hepatica*, the CatL proteases were also detected in the caecal lumen and within secretory vesicles of the caecal epithelia of juveniles and adult flukes using polyclonal anti-CatL antibodies (Collins et al., 2004). Therefore, the CatL may function as a protease for nutrition acquisitions in *Fasciola* sp., during late stages; and the early juveniles may employ its homolog (CatL1H) for nutritional acquisition as well as for invasion of the host's tissues.

The MoAb that we produced is quite specific to FgCatL1 and showed no cross-reactivity with antigens in other trematode, cestode and nematode parasites, including *P. cervi, E. pancreaticum, G. explanatum*, *S. spindale, S. mansoni*, *M. benedeni, A. centripunctata*, *Trichuris* sp., *H. placei* and *S. labiato-papillosa*. This impied that this MoAb might binds only to a common epitope, which is present exclusively in *Fasciola* cathepsin Ls. Hence, it is possible that this MoAb and its corresponding antigen could be used for immunodiagnosis of fasciolosis in the detection of both early and late infections with high sensitivity and specificity. Alternatively, the detection of other antigen such as tegumental antigen at MW 28.5 kDa and CatB3 by their corresponding MoAbs could also be employed in conjunction with the MoAb against CatL1, since these antigens were proven to be present and released from the adult and juvenile stages of *F. gigantica,* and their exploits in immunodiagnosis by sandwich ELISA have already been reported by our group (Anuracpreeda et al., 2009b, 2011).

2. Purification of the MoAbs and PoAbs against rCatL of *F. gigantica*

MoAb in culture supernatant and PoAb in serum specifically against rCatL were purified by precipitation in saturated ammonium sulfate solution. Thereafter, MoAb and PoAb were purified by a gel-filtration and ion exchange chromatography, respectively. Appropriate immunoglobulin fractions were collected and used for development of a monoclonal antibody (MoAb)-based sandwich ELISA. Hence, it is possible that this MoAb and PoAb could be used for immunodiagnosis for fasciolosis especially the detection of early and late infection.

3. Development of a MoAb-based sandwich ELISA for the detection of circulating CatL in serum of experimentally and naturally infected animals with fasciolosis caused by *F. gigantica*

The diagnosis of fasciolosis based on the detection of circulating parasite antigens in the body fluids of infected animals has been reported (Langley and Hillyer, 1989; Fagbemi et al., 1995; Viyanant et al., 1997; Velusamy et al., 2004). Antigen detection assay has several advantages over other methods as it can identify animals with pre-patent infection, which can not be detected by conventional parasitological test. It can also give a more accurate indication of current infection and the parasite load rather than past infection (Zheng et al., 1990). However, the detection of circulating antigen is more difficult since a defined antigen must be identified and this needs a more thorough investigation. In previous studies, the undefined *Fasciola gigantica* antigens at 26 to 28 kDa (Attallah et al., 2002) and 27 kDa (Kumar et al., 2008) have been used for the immunodiagnosis of fasciolosis. In the present study, I have shown that the well-defined CatL is a circulating antigen that could be detected in mice experimentally infected with *F. gigantica* were similar to the previous study. For example, Auracpreeda et al. (2009b) reported that MoAb against 28.5 kDa TA of *F. gigantica* could detect in WB, TA and ES of adult parasites as well as WB of metacercariae, newly excysted juveniles, 1, 4, and 7-week-old juveniles parasites. Also, Auracpreeda et al. (2013a) revealed that MoAb specific to rCatB3 of *F. gigantica* could detect both rCatB3 antigen and CatB3 in whole body of newly excysted juveniles and metacercariae of *F. gigantica*.

In the present study, a MoAb-based sandwich ELISA was utilized for detection of circulating *F. gigantica* CatL antigen in the sera from both *F. gigantica* experimentally infected mice and naturally infected cattle. The MoAb 4E3 against rFgCatL showed no cross-reactions with antigens of other adult parasites. Hence, I could use this MoAb 4E3

to detect the circulating CatL antigen in early and late stage of infection. In our earlier study, we have also produced MoAb against 28.5 kDa tegumental antigen (28.5 kDa TA) of adult *F. gigantica* and developed a sandwich ELISA for diagnosis of fasciolosis by *F. gigantica* in experimentally infected mice (Anuracpreeda et al., 2009b). Similarly, Viyanant et al. (1997) developed a MoAb-based sandwich ELISA for detection of circulating 66 kDa TA in the sera of cattle experimentally and naturally infected with *F. gigantica*. However, this MoAb cross-reacted with other parasite antigens and the MoAb clone was not stable. In addition, Fagbemi et al. (1997) produced MoAb against whole worm antigens of *F. gigantica* for the immunodiagnosis of fasciolosis in cattle. However, this MoAb exhibited more cross-reactivities with other parasite antigens. Likewise, Arafa et al. (1999) produced and utilized MoAbs against *F. gigantica* ES antigens for diagnosis of human fasciolosis. It was found that cross-reactivity with *S. mansoni* antigen occurred by using these MoAbs. In this study, our MoAb 4E3 was able to detect rCatL antigen and CatL in WB, ES, TA of *F. gigantica* at the concentrations as low as 3, 30, 40 pg/ml and 5 ug/ml, respectively. This could be attributed to the advantage of using the rabbit anti-mouse IgG to precoat the plate which helped to enhance the binding of MoAb, and hence also the binding to antigen. Furthermore, the use of MoAb as the antigen-capturing antibody increased the specific binding of the PoAb, which could help to reduce the background and yielded very low detection limits of the assay, which are lower than those reported by Langley and Hillyer (1989) who used a sandwich ELISA for detection of *F. hepatica* ES antigens in serum samples of experimentally infected mice at a concentration 0.25 ng/ml. As well, the detection limits are better than the results reported by Mezo et al. (2004) who used the monoclonal antibody (MM3) capture ELISA for the detection of *F. hepatica* ES antigens in fecal supernatants of infected animals, and found that the detection limit was 0.3 and 0.6 ng/ml in sheep and cattle, respectively. Likewise, Anuracpreeda et al. (2009b) had developed a MoAb-based sandwich ELISA for the detection of circulating 28.5 kDa TA in the serum samples from mice experimentally infected with *F. gigantica*. The result showed that the lower detection limit was 600 pg/ml for TA, 16 ng/ml for WB, and 60 ng/ml for ES antigens which are considerably higher than in this study. Taken together, Anuracpreeda et al. (2013a) used a MoAb-based sandwich ELISA for the detection of circulating CatB3 in the serum samples from mice experimentally infected with *F. gigantica*. The result revealed that the lower detection limit was 10, 100, and 400 pg/ml for rCatB3 antigen and CatB3 in whole body of newly excysted juveniles and metacercariae of *F. gigantica*, respectively, which are considerably higher than in this study. Also, the lowest detection limits reported herein are lower than that reported by Espino and Finlay (1994) who used

a sandwich ELISA to detect the ES antigens in stool samples of patients infected with *F. hepatica*, and found that the lower detection limit was 15 ng/ml, and that of Demerdash et al. (2011) who used a MoAb-based sandwich ELISA to detect the *F. gigantica* ES antigens in both serum and stool samples of patients at a concentration 3 ng/ml.

The sensitivity and specificity of this sandwich ELISA were also considered very high as in sera experimentally infected mouse these values were 95%, and 100%, while in naturally infected cattle sera they were 96.7% and 100%, respectively. Eventhough I do not know for how long animals in the field had been naturally infected, this finding indicated that this sandwich ELISA exhibited high efficiency in detecting circulating *F. gigantica* CatL antigen insera of naturally infected cattle. The sera from cattle infected with *F. gigantica* were collected from fields in many regions of Thailand which are endemic areas. The recurrence of infection with varying doses of metacercariae in natural cattle could occur. Hence flukes release varying amount of CatL antigen into the hosts' circulation and cattle were tested positive but with considerably different level of CatL. In addition, the diagnostic accuracy of this test in sera of naturally infected cattle was 98.9%, which is comparable to that of experimentally infected mouse at 98.5%. This finding indicated that this MoAb-based sandwich ELISA could be applied to naturally infected cattle with a large body size as well as in sera of small laboratory model animal such as mice as early as the first day postinfection. This early and late detection of CatL compared more favorably with other antigen-detection assays including that of Langley and Hillyer (1989) who used rabbit antiserum to detect circulating *F.hepatica* antigen in experimentally infected mouse sera at the 1st week post infection. As well, Viyanant et al. (1997) who could detect circulating 66 kDa TA in the sera of cattle experimentally infected with *F. gigantica* at the 1st week post infection. The later detections were also reported by Fagbemi et al. (1995) who could detect circulating 88 kDa *F. gigantica* antigen in sera of experimentally infected cattle at the 2nd and 3rd weeks after infection using a sandwich ELISA, and Velusamy et al. (2004) who could detect the circulating 54 kDa *F. gigantica* antigen in experimentally infected cattle sera atthe 2nd week after infection. Thus compared to these reports, our assay could detect the circulating CatL antigen at the earliest date so far. This is because CatL is among the antigens released early in NEJ stage and in relatively large amount (Meemon et al., 2010). In our study, the OD value reflecting the antigen level peaked at the 1st day post infection, and the level declined steadily to day 21post infection; then it became stable from days 21 to 35 in experimentally infected mouse. This indicated the rapid release of this antigen into the hosts' circulation. Later, the increased production of antibody in the host's circulation

might form the immune complexes, and reduced the amount of free CatL still available for detection. Alternatively, it is possible that the levels of the circulating antigen in infected sera were correlated with the number of worm still migrating in the hosts' tissues. During the first few weeks post infection, juvenile flukes are actively migrating throughthe host liver and they release copious amount of CatL into the host circulation. Once the parasites reached, became established and mature in the bile ducts, this antigen was released in much lower amount and into the bile ducts instead of the circulation, resulting in the lower level of the antigen in the latter. In summary, this study exhibited the efficiency of sandwich ELISA in detecting CatL antigen for early and late detection, which is better than the antibody detection which showed positive result very late. Hence, this MoAb-based sandwich ELISA for circulating CatL offers another promising and specific assay for fasciolosis, during the early and late infection.

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Output of the research

Fasciolosis due to *F. gigantica* is one of major tropical diseases that afflict cattle, buffaloes and sheep, as well as quite many human cases in the tropics. In Thailand, it causes serious economic loss in the order of 350-400 million bahts per year. The discoveries of efficient immunodiagnosis are essential for the detection, monitoring, for the early treatment with drugs, and for sustainable preventive measure from the disease. The present study provides basic knowledge on the nature of target antigens that have immunodiagnostic potential. The research could also yield specific monoclonal antibodies and antigens for the development of immunodiagnostic method for field applications in large economic animals in the future.

The main output is three scientific articles published in internationally reputable journals as following below:

1. **Anuracpreeda P*,** Chawengkirtikul R, Tinikul Y, Poljaroen J, Chotwiwatthanakun C, Sobhon, P. Diagnosis of *Fasciola gigantica* infection using a monoclonal antibody-based sandwich ELISA for detection of circulating cathepsin B3 protease. Acta Trop 2013; 127: 38-45. (IF = 2.787) **(*Corresponding author)**
2. **Anuracpreeda P***, Poljaroen J, Chotwiwatthanakun C, Tinikul Y, Sobhon P. Antigenic components, isolation and partial characterization of excretion-secretion fraction of *Paramphistomum cervi*. Exp Parasitol 2013; 133: 327-333. (IF = 2.154) **(*Corresponding author)**
3. **Anuracpreeda P***, Srirakam T, Pandonlan S, Changklungmoa N, Chotwiwatthanakun C, Tinikul Y, Poljaroen J, Meemon K, Sobhon P. Production and characterization of a monoclonal antibody against recombinant cathepsin L1 of *Fasciola gigantica*. Acta Trop 2014; 136: 1-9. (IF = 2.787) **(*Corresponding author)**

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