APPLICATION OF ISO-ELECTRIC FOCUSING *BITHYNIA* SNAIL ANTIGEN FOR ELISA IgG-SUBCLASS DETECTION OF HUMAN OPISTHORCHIASIS

WALLOP PAKDEE

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……..…….
Mr. Wallop Pakdee
Candidate

……..... ……
Assoc. Prof. Paron Dekumyoy, Ph.D.
Major Advisor

.....……
Assoc. Prof. Jitra Waikagul, Ph.D.
Co-Advisor

…….. ……..…….……
Assist. Prof. Thareerat Kalambaheti, Ph.D.
Co-Advisor

……..……
Assoc. Prof. Banchong Mahaisavariya, M.D.
Dean
Faculty of Graduate Studies
Mahidol University

……..……
Assoc. Prof. Malinee Thairungroj, Ph.D.
Chair
Master of Science
Program in Tropical Medicine
Faculty of Tropical Medicine
Mahidol University

Thesis
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on
April 20, 2009

……..……
Mr. Wallop Pakdee
Candidate

…….. ……
Assoc. Prof. Duangduen Krailas, Ph.D.
Chair and External examiner

……..……
Assoc. Prof. Paron Dekumyoy, Ph.D.
Member

……..….. ……
Assis. Prof. Thareerat Kalambaheti, Ph.D.
Member

…….. ……
Assoc. Prof. Jitra Waikagul, Ph.D.
Member

……
Prof. Banchong Mahaisavariya, M.D.
Dean
Faculty of Graduate Studies
Mahidol University

……
Assos. Prof. Pratap Singhasivanon, M.B.B.S.,
D.T. M. & H. (Bangkok),
Dr.P.H. (Epidemiology)
Dean
Faculty of Tropical Medicine
Mahidol University

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Wallop Pakdee

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WALLOP PAKDEE 4937698 TMTM/M

M.Sc. (TROPICAL MEDICINE)

THESIS ADVISORY COMMITTEE: PARON DEKUMYOY, Ph. D., JITRA WAIKAGUL, Ph.D, THAREERAT KALAMBAHETI, Ph.D.

ABSTRACT

A diagnosis of opisthorchiasis may be confirmed by the characteristic eggs and worms in feces or other clinical specimens, and genetic materials. However, misdiagnosis using these techniques may occur in light infections, and where the eggs are morphologically similar to those of other parasite species. A finding of immune mediators can help to confirm the type of infection. Therefore, this study used indirect ELISA to detect total IgG and IgG subclasses with partially purified *Bithynia siamensis goniomphalos* antigens, which were prepared by liquid-phase iso-electric focusing using Rotofor cells. Twenty isoelectric focusing fractions of snail antigens were first analyzed for a potential antigenic candidate, using OD-ELISA ratio comparison. The selection of iso-fractionated antigen (Iso-FAg) for ELISA was based on a high OD-ELISA ratio between pool-positive and pool-negative. Iso-F antigens 7, 7, 6, 2, and 10 gave high OD-ratios to total IgG, IgG1, 2, 3, and 4, respectively. A full-scale ELISA was then conducted with serum samples from 50 opisthorchiasis cases, 196 other parasitic-disease cases, and 35 healthy controls. Only Iso-FAg 7 to IgG1 showed the best potential antigen for detecting opisthorchiasis, with 98% sensitivity and 89.17% specificity at cut-off point 0.221. Total IgG detection showed lower sensitivity (96.00%) and specificity (51.94%) at cut-off 0.498. However, low cross-reactivity to IgG1 was found for gnathostomiasis (2/12), trichinellosis (1/10), Bancroftian filariasis (1/10), enterobiasis (1/10), neurocysticercosis (3/10), taeniasis (2/10), echinococcosis (1/10), sparganosis (2/3), paragonimiasis heterotremus (3/10), schistosomiasis (2/10), fascioliasis (2/4), and haplorchiasis (3/10). It is summarized that Iso-FAg7 to IgG1 is a good candidate antigen to antibody against *O. viverrini* but not for cross-reactivity. Based on these results, Iso-FAg7 to IgG1 can be re-analyzed by cross-reactive sera encountering opisthorchiasis antibody, by comparing antigen-antibody banding; then, only antigens reactive to opisthorchiasis will be selected and then electroeluted from a polyacrylamide gel as an eluted antigen. Another method to provide an antigen, Iso-F antigen candidates can be selected by reacting all fractioned antigens with opisthorchiasis antibody, indicating no, or lesser, reaction with healthy controls; the selected antigens may be pooled to create a cocktail antigen.

KEY WORDS: *BITHYNIA SIAMENSIS GONIOMPHALOS* ANTIGENS/LIQUID-PHASE ISO-ELECTRIC FOCUSING/ OPISTHORCHIASIS/ IgG1-4-ELISA

114 pages

การใช้แอนติเจนจากหอย *Bithynia* ที่เตรียมด้วยวิธี Iso-electric focusing เพื่อตรวจหา IgG-subclss ของโรคพยาธิใบไม้ตับในคนโดยวิธี ELISA

APPLICATION OF ISO-ELECTRIC FOCUSING *BITHYNIA* SNAIL ANTIGEN FOR ELISA IgG-SUBCLASS DETECTION OF HUMAN OPISTHORCHIASIS

วัลลภ ภักดี 4937698 TMTM/M

วท.ม. (อายุรศาสตร์เขตร้อน)

คณะกรรมการที่ปรึกษาวิทยานิพนธ์: พารณ ดีคำย้อย Ph.D., จิตรา ไวคกุล Ph.D.,ธารีรัตน์ กะลัมพะเหติ, Ph.D.

บทคัดย่อ

การวินิจฉัยโรคพยาธิใบไม้ตับอาจได้รับการยืนยันจากการดูลักษณะของไข่และตัวพยาธิในอุจจาระหรือสิ่งส่งตรวจอื่นและยังอาจใช้ตัวอย่างทางพันธุกรรมก็ได้ อย่างไรก็ตามความผิดพลาดในการวินิจฉัยอาจเกิดขึ้นจากการติดเชื้อเพียงเล็กน้อยและรูปร่างของไข่ที่คล้ายกับไข่พยาธิใบไม้ชนิดอื่น ส่วนการตรวจหาภูมิคุ้มกันต่อโรคก็สามารถนำมาช่วยได้ ดังนั้นการศึกษานี้จึงใช้วิธี indirect ELISA เพื่อตรวจหา total IgG และ IgG subclass โดยใช้แอนติเจนจากหอย *Bithynia siamensis goniomphalos* ที่เตรียมโดยวิธี liquid-phase isoelectric focusing ด้วยเครื่อง Rotofor cells และแอนติเจน (Iso-FAg) ทั้งยี่สิบชนิดได้นำมาวิเคราะห์เพื่อหาแอนติเจนทางเลือกโดยการหาค่าสูงสุดของ OD-ELISA ratio ระหว่างซีรั่มโรคพยาธิใบไม้ตับและคนปกติพบว่า Iso-FAgs 7, 7, 6, 2, และ 10 ที่มีผลต่อการตรวจหา total IgG, IgG 1, IgG 2, IgG 3, และ IgG 4, ตามลำดับสามารถนำไปวิเคราะห์ต่อด้วยซีรั่มโรคพยาธิใบไม้ตับ (50 ราย) โรคอื่น ๆ (196) คนปกติ (35) จากการทดสอบ Iso-FAg 7 ต่อ IgG1 ให้ผลดีที่สุดด้วยค่า 98% sensitivity และ 89.17% specificity ส่วนการหา total IgG ผลจะต่ำกว่าทั้ง sensitivity (96%) และ specificity (54.11%) แต่ปฏิกริยาข้ามต่อ IgG1 นั้นพบว่ามีโรคพยาธิตัวจี๊ด (2/12) โรคทริชิเนลโลซิส (1/10) โรคเท้าช้าง (1/10) โรคพยาธิเข็มหมุด (1/10) โรคพยาธิเม็ดสาคูหมู (3/10) โรคพยาธิตัวตืด (2/10) โรคเอคไคโนคอคโคซิส (1/10) โรคพยาธิตืดปลา (2/3) โรคพยาธิใบไม้ปอด (3/10) โรคพยาธิใบ ไม้ในเลือด (2/10) โรคพยาธิฟาสซิโอล่า (2/4) และโรคพยาธิใบไม้ในลำไส้ (3/10) สรุปได้ว่า Iso-FAg 7 ต่อ IgG1 นั้นสามารถนำไปใช้ในการตรวจจับแอนติบอดีต่อพยาธิใบไม้ตับได้แต่จากปฏิกริยาข้ามนี้คิดว่า Iso-FAg 7 น่าจะนำมาวิเคราะห์ใหม่เพื่อดึงเอาแอนติเจนจำเพาะต่อการวินิจฉัยโรคพยาธิใบไม้ตับ

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LIST OF ABBREVIATIONS

BSA	bovine serum albumin
°C	degree celsious
cm	centimeter
DW	distilled water
ELISA	enzyme-linked immunosorbent assay
et al.	et alli
ES	excretory-secretory
FN	false negative
FP	false positive
g	gram
hr	hour
i.e.	id est
IgG	immunoglobulin G
kg	kilogram
mA	milliampere
mg	milligram
min	minute
ml	milliliter
M	mole
MW	molecular weight
µl	microliter
µm	micrometer
nm	nanometer
OD	optical density
PBS	phosphate buffer saline
PBS-T	PBS with 0.05% tween-20

LIST OF ABBREVIATIONS (cont.)

pH	negative logarithm of hydrogen-ion activity
pI	isoelectric point
%	percent
rpm	round per minute
SDS	sodium dodecyl sulphate
TN	true negative
TP	true positive
w/v	weight per volume
$\bar{x}$	mean

CHAPTER I
INTRODUCTION

The fish-borne zoonoses are described for a large number of human infections, obtained through consumption of raw fish such as opisthorchiasis, intestinal trematodiases, and diphyllobotriasis (Chai *et al.*, 2005). In southeast Asia, opisthorchiasis and heterophyid intestinal trematodiases are the major problem (Sithithaworn and Haswell-Elkin., 2003; Chai *et al.*, 2005) in the case of similar morphology, and are lead to misdiagnosis. The opisthorchiasis is associated with a member of hepatobiliary disease, including cholangitis, obstructive, jaundice, hepatomegaly, cholecystitis, and cholelithiasis (Harinasuta *et al.*, 1984). Although heterophyid intestinal trematodiases are serious problem, several heterophyid species, S*tellantchasmus falcatus*, *Haplorchis* spp. and *Procerovum* spp., can cause significant pathology in the heart, brain, and spinal cord of humans (Africa *et al.*, 1940; WHO, 1995). The World Health Organization (1995) has estimated that the members of people currently infected with fish-borne trematodes exceeds 18 million, including those live in developed countries, is more than half a billion (WHO, 1995).

Liver fluke infections distributed in southeast Asia, in particular the Greater Mekong Subregion. Nine million people with *Opisthorchis viverrini* infection are prevalent in Thailand, Lao PDR, Vietnam and Cambodia, while 7 million people with *Clonorchis sinensis* infection is prevalent in Vietnam (WHO, 1995). Recently, the first finding of *C. sinensis* is reported in Chachoengsao Province, East, Thailand (Traub *et al*., 2009). In Lao PDR, Mekong River Basin is the most heavily infected area (Chai *et al*., 2005). In Vietnam, several southern provinces such as Phu Yen, have reported *O. viverrini* infection above 10% (De *et al.*, 2003). In Thailand, there was 7 million Thai people infected, and percent of *O. viverrini* infection was 6.7% in 2000, while this percent was increased to 9.4% in 2001 (Jongsuksuntigul and Imsomboon, 2003). It is the most prevalence in northeast and north regions in Thailand (Preuksaraj, 1984;

Sukontason *et al.,* 1999; Jongsuksuntigul, 2002). The number of infected people in the northern region alone was estimated in 1960s to be over 3.5 million (Wykoff *et al.,* 1965). This figure seems to change; the estimated number of infected people is currently about 6 million (Sripa *et al.,* 2002; Chai *et al.*, 2005).

Among freshwater fish, there 186 susceptible species on Family Cyprinoidae, that are a second intermediate host in a life cycle of *O. viverrini* for the developmental stage of metacercaria. The raw or undercooked fish are source of human infection, When someone ingests them the metacercariae are digested by gastric and intestinal juice pass through the duodenum to the bile duct (Harinasuta *et al.,* 1984; WHO, 1995; Waikagul, 1998). The metacercariae develop to the adult stage in about 4 weeks after infection (Rim, 1986). The adult worm parasites can cause hepatobiliary disease and tend to obstruct the bile duct that is risk factor for chlolangiocarcinoma (Harinasuta, 1978; Pinlaor *et al*., 2004; Sriamporn *et al*., 2004). The standard diagnosis method to detect liver fluke infection is microscopic examination of stool to observe morphological characteristics of egg (Harinasuta *et al.,* 1984; WHO, 1995). However, it is not straight forward: only an expert is likely to give an accurate opinion (Waikagul, 2002). Since, the *O. viverrini* egg is similar in size and shape with other small liver flukes and small intestinal flukes such as *C. sinensis*, *O. felineus*, and *Haplorchis* spp. (Ditrich *et al.,* 1992; Waikagul *et al*., 2002). For *O. viverrini*, the morphology of adult worm is very similar to that of *O. felineus* and the eggs are slightly elongated ovoid in shape, and thus difficult to distinguish from other members in the same family (Rim, 1982). Thus, it tends to cause false-positive in stool examination.

Recently, many researchers have attempted developing the methods for diagnosis of the small liver flukes, such as molecular biological and immunological methods (Waikagul *et al*. 2001, 2002; Parvathi *et al.,* 2007; Thaenkham *et al.,* 2007). Serodiagnosis is an alternative diagnostic tool under knowledge to detect antibodies against *O. viverrini* in the sera of infected individuals. The concept of immunodiagnosis is the relationship between antigen and antibody in specific reaction. Several developed antigens have been produced from simple extractions to

complicated techniques using adult worms, larval stages, excretory-secretory antigen (ES-antigen) and snail intermediate hosts and evaluated for their specificity in detecting opisthorchiasis antibody (Srivatanakul *et al*., 1985; Wongratanacheewin *et al.*, 1988; Amornpunt *et al*., 1991; Sirisinha *et al*., 1991, 1992, 1995; Akai *et al.*, 1995; Sakolvaree *et al*.,1997; Watthanakulpanich *et al*.,1997; Sripa and Kaewkes, 2000; Wongsarojt *et al*.,2001; Waikagul *et al*., 2002).

Based on snail host-parasite relationship, miracidia of trematodes can finally develop into cercarial in highly susceptible snail intermediate hosts; it means that those parasites may have a possible mechanism to avoid host immune recognition. They may produce their structural similarity to those of snail's structures, which protect themselves from snail's immune system (Damian, 1964; 1979). In contrast, snails' host defense can make encapsulation of incompatible trematode larvae in abnormal snail hosts by leukocytes or fibers surrounding and subsequently parasites are degenerated (Newton, 1952; 1954; Brooks, 1953; Sudds, 1960). This evidence can also be found in detection of host defense, *Trichobilharzia elvae* miracidia penetrate to two abnormal hosts, *Bulimnaea magasoma* and *Fossaria abrussa*, and then die within 2-6 days (Sudds, 1960). Therefore, several studies have been reported the shared antigens between trematodes and their snail intermediate hosts for serodiagnosis of parasitic diseases. Serodiagnosis of human schistosomiasis, which has been difficult in producing mass schistosome antigens can be succeeded with alternative antigens from snail intermediate hosts (Fairly, 1919; Jackson and DeMoor, 1976; Rivera-Marrero and Hillyer, 1985; Markl *et al*., 1991; Alves-Brito *et al*., 1992; Li *et al*., 1997). In the early of such studies, Yoshino and Cheng (1978) reported that newly hatched miracidia of *S. mansoni* possess surface membrane associated determinants antignically similar to components of the haemolymph of the intermediate snail host, *Biomphalaria glabrata*. It was found that three antigens were demonstrated to be shared between adult *Schistosoma mansoni* and its molluscan intermediate host. The antigen was shown to strong reaction with sera of schistosome infected mice and humans. Another study on schistosomiasis, antigen from *Oncomelania* snail intermediate host show a number of reactive bands with antibodies from immunized with extracts of adult *S. japonicum* worms using immunoelectrophoresis but snail

extracts from other non-host genera give no reaction (*Lymnaea*, *Biomphalaria*, *Pila*, and *Semisulcospira*) or only one band by *Bulinus* snail Tsuji *et al*., 1978). An alternative antigen of *B. glabrata*, which is snail intermediate host of *Angiostrongylus* (*Parastrongylus*) *cantonensis* show two reactive bands (24 and 48 kDa) with sera of parasitologically confirmed angiostrongyliasis and in-reactive to other parasitic infections (Eamsobhana *et al.*, 1999).

Therefore, a proteomic part of snail antigens, which acts as antigen has been found out and analyzed for opisthorchiasis detection. Then, in the study on comparison among extracts of three species of *Bithynia* snails, *B. siamensis siamensis* from Bangkok, *B. siamensis goniophalos* from Khon Kaen province, and *B. funiculata* from Lampang province, showed distinctly similar complex protein profiles and also major similarity of proteins of adult *O. viverrini* worms by SDS-PAGE using Coomassie brilliant blue staining (Watthanakulpanich, Thesis, 1995; Watthanakulpanich *et al.*, 2002). Other species of bithyniid snails, *Parafossarulus manchouricus, B. tentaculata* and *B.* (*Gabbia*) *misella* also show similar protein patterns (Chung, 1984). The mimic antigens of snail intermediate hosts have been established in detection of opisthorchiasis using *B. funiculata*, *B. siamensis siamensis*, and *B. siamensis goniomphalos* snails (Wathanakulpanich *et al*., 1997, Waikagul *et al.*, 2002). When comparing between antigens of *O. viverrini* worms and these snails, majority of these snail extracts are reactive to opisthorchiasis antibodies of human cases and also identical to several bands of *O. viverrini* antigen (Watthanakulpanich, Thesis, 1995; Watthanakulpanich *et al.*, 2002). The whole snail extract of *B. funiculata* gives the best result as good as adult *O. viverrini* antigen by IgG-ELISA for human opisthorchiasis. The evaluation results, sensitivity and specificity are 80.2% and 81.2%, respectively. However, cross-reactions of this antigen are occurred with paragonimiasis and strongyliodiasis (Wathanakulpanich *et al*., 1997). For obtaining the better results using two different techniques, the crude antigens from *B .s. goniomphalos* are electroeluted at 53 kDa from polyacrylamide gel as eluted antigen. This band is the prominent reactive to opisthorchiasis antibody, very low cross-reactivity to other parasite infections and not to normal controls. A cocktail antigen is selected from antigenic fractions, which prior separated by Sephacryl S-200 HR gel

filtration chromatography. Both antigens are analyzed by IgG-ELISA. The eluted antigen gives result of specificity and sensitivity at 98.4% and 98.0%, respectively, and the cocktail antigen shows specific and sensitive results at 88% and 88.5%, respectively. Cross reactions with eluted antigens are occurred with hymenolepiasis and strongyloidiasis (Waikagul *et al*., 2002). It seems that more purification of crude antigen is required to produce higher sensitivity and specificity of tests. Fortunately, the snail is cost less than adult *O. viverrini* maintenance, which is needed to collect metacercariae and grown into adult worms in animal hosts and the large amount of snail's material can be obtained rapidly (Chanawong *et al*., 1990). Waikagul *et al.*, (2001) showed that *Bithynia* snail antigen could be used to replace worm antigen for antibody detection of *O. viverrini* in human.

Detection of immunoglobulin G is the main point of antigen-antibody reaction in the field of helminthic infections. A more interesting reaction is to detect subclasses of IgG that IgG1, IgG2, IgG3, and IgG4 can differentiate and be successful in specific detection on focusing research and diagnosis, such as filariasis (Ottesen *et al*., 1985; Chanteau *et al*., 1991; Egwang *et al.*, 1991) strongyloidiasis (Genta and Lillibridge, 1989) trichuriasis (Lillywhite *et al.*, 1991) chronic schistosomiasis (Iskander *et al*., 1981; Jassim *et al*., 1987) hookworm infection (Loukas *et al.*, 1996), angiostrongyliasis (Intapan *et al.*, 2003), paragonimiasis (Wongkham *et al*., 2005), gnathostomiasis (Nuchprayoon *et al*., 2003; Anantaphruti *et al*., 2005; Laummaunwai *et al*., 2007). From those researches, Wongkham *et al*. (2001) reported that the detection of IgG4 antibodies to an excretory-secretory (ES) product of *Paragonimus heterotremus* at 31.5 kDa is the most reliable test by immunoblotting. The result showed accuracy, sensitivity, specificity, and positive and negative predictive values of 97.6%, 100.0%, 96.9%, 90.0% and 100.0%, respectively. When the complicated somatic antigen, especially adult worm extracts, is used in the detection, subclass IgG antibodies give a satisfied result. Intapan *et al*. (2003) reported that the adult somatic antigen recognition pattern for IgG subclass (IgG1, IgG2, IgG3, and IgG4) was different among the molecular protein masses scattering from less than 14.4 to more than 116 kDa by immunoblot. The IgG4 of human angiostrogyliasis sera specifically recognized 29 kDa antigen and the reaction gave the highest specificity and

sensitivity, 95.0% and 75.0%, respectively. For gnathostomiasis detection, anti-*Gnathostoma spinigerum* IgG1 antibody provided the highest sensitivity (98.0%), whereas anti-*G. spinigerum* IgG2 antibody detection gave the highest specificity (88.0%) when crude antigen was used by IgG subclass ELISA (Nuchprayoon *et al*., 2003). Anantaphruti *et al*. (2005) reported that the 21 kDa antigenic band of *G. spinigerum* larval extract recognized faintly by IgG4 antibody from human gnathostomiasis sera. The result of 21 kDa band showed 100.0% sensitivity and 100.0% specificity, whereas the 24 kDa band indicated 92.9% sensitivity and 93.4% specificity.

In serodiagnosis, an effective antigen is required for high evaluation of test. ELISA needs such antigen, i.e., partially purified and purified antigens. It is opposite to immunoblotting technique, which crude antigens are separated into several fractions in gel. To use a higher technique in obtaining a good antigen, iso-electric focusing (IEF) technique by liquid-phase can provide separated antigens following pH gradient in solution. Using Rotofor-separation (Bio-Rad), liquid-phase iso-electric focusing, the non-charge antigens of cystic fluid, (fraction no.18) and intact cyst (fraction no.19) of *Taenia solium* metacestodes showed 100% sensitivity and specificity by ELISA (Ito *et al*., 1998). Previous studies indicated improvement of the purified antigens for characterization and diagnosis of onchocercosis (Klenk *et al*., 1983). Fluid antigen and crude cystic antigen of *Taenia solium* matacestodes were separated by liquid-phase iso-electric focusing to be 20 fractioned antigens. The fractioned antigens Nos. 9-15 of crude cystic antigen showed good result in detection of cysticercosis antibody in pig sera. The result gave negative control by ELISA method with all the other antisera against common parasites of Chinese pigs, and the fractioned No. 15 was the best candidate antigen shown the highest ratio between positive and negative of OD-ELISA (Ko and Ng, 1998).

Thus, this study, we would like to scope:

1 Source of antigen: *Bithynia s. goniomphalos* snail intermediate host was used because the snail distributes in the endemic areas of opisthorchiasis in the northeastern

provinces, the high antigenic quality, lower cost of maintenance and large amount of antigenic materials (Chanawong *et al*., 1990; Waikagul *et al*., 2002).

2. Method of antigen preparation: Iso-electric focusing (IEF) (Rotofor® System, Bio-Rad) liquid phase was used in separation of crude antigen.

3. Analysis of antigen: IEF antigens were applied in the IgG-ELISA and emphasizing in IgG-subclass ELISA to detect opisthorchiasis antibody in infected human and control sera of heterologous infections and healthy donors.

CHAPTER II
OBJECTIVES

1). To prepare fraction of partially purified antigens from *B. s. goniomphalos* snail extract by liquid phase iso-electric focusing method.

2). To evaluate IEF antigens by IgG-ELISA and emphasizing IgG subclass ELISA system to detect specific antibody of opisthorchiasis in human encountering other parasitic infections and healthy controls.

CHAPTER III
LITERATURE REVIEW

1. Epidemiology of small liver flukes

Three species of food-borne trematodes, *Opisthorchis viverrini*, *Clonorchis sinensis*, and *O. felineus*, are important because of the high incidence of chlolangiocarcinoma and hepatobiliary diseases (Sithithaworn *et al*., 2003). It remains a major public health problem in many countries of Eastern Europe, the Far-East and Southeast Asia (IARC, 1994). In 1995, 17 million people in worldwide infected by these three species approximately dividing into 7 million people infected with *C. sinensis*, 9 million people with *O. viverrini*, and 1.2 million with *O. felineus* (Preuksaraj, 1984; Rim, 1986; WHO, 1995). Different geographical regions present both same and different species based on many suitable conditions to life cycle of the parasites. For example, *C .sinensis* is endermic in China, Korea, Taiwan, Vietnam, and Japan, *O. felineus* is prevalent in Rusian federation and eastern Europe, and *O. viverrini* is important in southeast Asia, such as Lao PDR, Cambodia, and Thailand (Sithithawarn and Haswell-Elkins, 2003).

In Thailand, there are 7 million people or representing approximately 15% of Thais infected with *O. viverrini*. This parasite is the most prevalence in north eastern region, especially in Khon Kean province. 1.73 million people were infected with *O. viverrini*. However, in northern Thailand, 16 % of total population in this area was also infected. Normally, a patient infected with *O.viverrini* is also infected with minute intestinal fluke. These, in stool examinations, were usually found mixed eggs between *O. viverrini* and *O. viverrini* -like egg (Jongsuksuntigul and Imsomboon, 2003; National Statistics Office, 2000).

In 1998, Radomyos and colleagues reported 431 residents from 16 provinces in northern Thailand who has previously been found positive of *O. viverrini* or *O.*

viverrini-like eggs after 40 mg/kg praziquantel treatment. The stools were collected for 4 to 6 times and examined for adult worms. The prevalence of *O. viverrini* in this group was 11.6 % while intestinal flukes, *Haplorchis taichui* and *H. yogokawai*, were predominantly found in 63.11 % and 10.44 %, respectively.

2. *Opisthorchis viverrini*

O. viverrini is classified under Phylum Phatyheminthis, class Trematode, order Prosostomate, and Family Opisthorchiidae (Chatterjee, 1980). The adult worms usually inhabit in distal bile duct of human host although some may reside in the gall-bladder of man and animals (Wykoff *et al*., 1965). Adult worm is slender, flat, transparent, reddish-yellow, and lancet shape. The average size of body is 5.5-9.5 mm in length and 0.7-1.6 mm in width (Harinasta, 1969; Malek, 1980). The parasite is belonged to Digenea, in which an asexual multiplication of the larval stage in snail and fish, and followed by sexual reproduction in adult stage of human host. The life span of *O. viverrini* is 15-20 years (Belding, 1965) and the prepatent period takes about 22-30 days when the eggs can be found in the feces of infected hosts (Wykoff and Ariyaprakai, 1966). The embryonated egg of *O. viverrini* is small, oval flask-shaped, yellowish-brown, and operculum that has a distinct shoulder and measures 22-32 x 11-12 μm in average size. The outer shell is ridge-like appearance forming irregulator pattern (Ishily, 1972). The egg contains a fully developed miracidium when passed in stool (Sadun, 1995; Malek, 1980). After *Bithynia* snail ingests, eggs hatch in the gut, release miracidia, which develop to be the mother sporocysts (size mature stage, 1.1 x 0.65 mm), redia (size, 0.18 x 1.1 mm in length and 0.08 x 0.28 mm in width) and finally cercarial stage (207 μm in length) (Wykoff, *et al.,* 1965). Cercaria emerges from the *Bithynia* snail that is the first intermediate host. The *O. viverrini* cercariae are found to vary in total length of 450-595 μm the mean length of the body is 154 μm, while the tail is 6 μm in length.

O. viverrini metacercaria, the infective stage, develops after cercaria from snail penetrating in various species of fresh-water cyprinoid fish (Harinasuta and Harinasuta, 1984; WHO, 1995; Waikagul, 1998). The cyst is usually oval and measured 204 x 145 μm. There are two thin walls and a surrounding fibrous capsule,

about 15-30 μm, formed by host-tissue reaction. The larva within the cyst shows vigorous movement. Normally, it can be seen the inner organs under electron microscope such as oral, and ventral suckers (a group of white granules) and observed in excretory bladder of the larva (Harinasuta, *et al*., 1957; 1960).

3. The snail: first intermediate host

Three species of aquatic snails in the family Bithyniidae is the first intermediate host of *O. viverrini* liver fluke in Thailand. There are *Bithyiai funiculata, B. siamensis siamensis, B. s goniomphalos* (Harinasuts, 1969). In Thailand, *B. fumiculata* acts as the snail intermediate host in the northern part, *B. s siamensis* in the central part, and *B. s goniomphalos* in the northeastern part, respectively (Brandt, 1974). The size of individual snail is about 9-11 x 5-7 mm. *Bithyiai* snails become mature and produce offsprings for 6 month old. For young snails, 1-3 month old, are more susceptible than older snails. The life span of this snail is approximately 2 years (Chanawong and Waikagul, 1991). In 1991, Chanawong evaluated the infection rate of *Bithynia* snail with *O. viverrini* eggs and it was found that 50 miracidail eggs per snail yielded the highest percentage of living surviving positive snails (Chanawong and Waikagul, 1991).

4. *B. siamensis goniomphalos*

Normally, *B. s.goniomphalos* snails are infected with *O. viverrini* in rainy and winter seasons with the monthly infection rates ranging from 0.03% to 0.36% (Lohachit, 2001). In the laboratory infection, *B. s.goniomphalos* can give an infection rate of 9.6% (Chanawong and Waikagul, 1991). These studies indicate that even though these snails are determined to distribute widely in the liver fluke endemic area, the infection rates of *O. viverrini* cercariae in the snails are rather low in both natural phenomenon and experimental condition.

The external feature of *B. s.goniomphalos* snail can be identified by its shell morphology. The shell is dull and normally reddish-brown in color with 10.5 mm in length and 6.5 mm in width on average. This conic shell has small rounded whorls with horizontal and indented sutures. Actually, the outer part of the last whorl is rather

straight, but this apex is found very much eroded when the snail becomes older. The shell surface consists of thick transverse raised lines and fine spiral incised lines. The umbilicus is relatively wide and deep, and the basal lip of the aperture has a sharp angle on the left side (Chitramvong, 1992).

The *B. s.goniomphalos* prefers to reside in the standing water such as reservoirs, ponds, and rice fields rather than flowing water in the rivers, and it is frequently found on and in a sandy substrate mixed with mud along the edge of a pond that is rather deep and lacking in algae or water plants growing along the shore (Chitramvong, 1992). The maximum of the snail population density occurs naturally during the cold season; in addition, in the same habitat, many species of other snails are usually found (Lohachit, 2001). As a result, laboratory bred snails are probably more convenient to use in this study than the field snails that may cause difficulty in differentiating *B. s.goniomphalos* from those other species of snails.

5. The fresh water fish: second intermediate hosts

The second intermediate host of the parasite is fish in the family Cyprinidae. They are the major intermediate host of *O. viverrini* in three genera: *Cyclochilichthys* (*C. apagon, C. armatus, C. siaji*) *Puntius* (*P leiacanthus, P. orphoides P .viehoever P. paripentarona*) and *Hampata dispa,* from which the mature infective metacercaria are recovered (Wykoff *et al*., 1965; Viranuratti and Stituimankarn, 1972; Cheng, 1973). *O.viverrini* metacercariae are found that in various parts of the fish body. The highest density is in the body muscle, followed by the dorsal, pectoral, pelvic, and anal fin muscles, respectively (Vichasri *et al*., 1982; Kaekes 2003).

6. Infection in intermediate host and reservoir host

For *O. viverrini*, eggs are reached a body of freshwater are ingested by an appropriate snail (*Bithynia* spp.) (Brockelman, *et al*., 1986; Ditrich *et a*l., 1990), and developed to be sporocysts. The sporocysts produce thousands of redia every daily, approximately 2 months after snail infection. The free-swimming cercariae shed their tail, penetrate the tissue of host fish and encyst; becoming a fully infective metacercaria in 21 days. About 80 species of the cyprinoid family and at least 13

species of other species serve as secondary intermediate host (Komiya, 1966; Vichasri, *et al*., 1982; Rim, 1986; WHO, 1995). The life cycle requires at least 4 months completing (Rim, 1986). The prevalence of liver fluke infection in snail intermediate hosts is typically low (range 0.05-0.07) in *Bithynia* spp. (Harinasuta, 1969; Brockelman *et al*., 1986). Snail population exhibits dependent on rainfall and paddy fields, but rapidly disappear over the dry season (Brockelman *et al*., 1986). However, the prevalence of infected fish is much greater than snail. 90% to 95% of cyprinoid fish species harbor *O. viverrini* metacercariae (Harinasuta and Vajrasthira, 1960; Vichasri *et al*., 1982). The most common species of cyprinoid fish were *Puntius*, *Cyclocheilichthys* and *Hampala* (Wykoff *et al*., 1965). The intensity of liver fluke infection in fish varies by season, type of water body, species and individual (Vichasri *et al*., 1982).

Transmission of fish and subsequently human may be highest after the peak of monsoon flooding, when snails and fish are abundant (Vichasri *et al*., 1982; Haas, *et al*., 1990). The frequency of infection in reservoir hosts like pigs, cats, rats and dogs varies by areas and is not closely associated with human infection (Chai *et al*., 2005).

For heterophyid intestinal flukes (HIFs), the life cycle, transmission and mode of infection are like opisthorchiid liver flukes (OLFs). The first intermediate host of HIF group is *Melanoides* spp. The reservoir hosts are cats, dogs, etc. (Velasques, 1982).

7. Source of human infection

Although most published descriptions of social habits regarding consumption of raw fish is careful sociological investigation, raw or undercooked fish is the primary vector of infection (Sithithaworn and Haswell-Elkin, 2003). Several studies indicate the survival of infective stages depends on the concentration of salt and degree of fermentation (Vichasri, 1981; Tesana *et al*., 1986). Koi pla is probably the most infective, followed by fish preserved for over 7 days, then Pla ra and Jaewbhong (Sithithaworn and Haswell-Elkins, 2003).

8. Pathobiology

The clinical features of opisthorchiasis are classified into 4 types depending upon the intensity and duration of the infection. The infection may be either 1) an asymptomatic type in which there may be only intermittent abdominal dull pain with slight discomfort, 2) a mild type with irregular episodes of flatulence and dyspepsia and “a hot sensation” over the liver area, 3) a moderate type with symptoms of mild cholangitis, dyspeptic flatulence, diarrhea or 4) a severe type with chronic relapsing cholangitis, cholecystitis, choletithiasis and obstructive jaundice which are a main risk factor for chlolangiocarcinoma (CHCA) (Harinasuta,1978,1984). The main *O. viverrini* infection is occurred in liver, extra-hepatic bile duct and gall bladder in both human and experimental animals (Tansurat, 1971; Bhamarapravati *et al*., 1978; Koompirochana *et al*., 1978; Harinasuta *et al*., 1984; Ringantti *et al*., 1989) and when a proportion of heavily infection people of adult worm parasite may cause hepatobiliary disease. It is not clear whether worms killed by the praziquantel contribute to gall stone formation or increase the risk of CHCA with repeated treatment (Sripa, 2003; Pinlaor, 2004; Sriamporn, 2004).

9. Prevention and control strategies

Since the traditional human habit of eating raw or improperly cooked freshwater fish is a major reason for sustaining the zoonosis in endemic area. Although health education efforts aimed at changing such habits, the people still like to eat in the same way (Guoqing *et al*., 2001; WHO, 2004). This is an intractable obstacle to control fish borne trematode infection, especially OLFs and HIFs. Currently, the major strategies for community prevention and control include fecal examination and treatment of individual cases with praziquantel (25 mg/ kg, three times daily for 2-3 days), giving health education to change habit in eating improper food, and environmental sanitation through the building and use of latrines in endemic areas (Chai *et al*., 2005). However, WHO (2004) has recommended mass chemotherapy of people at risk in endemic areas, but long-term treatment of this approach may be problematic (Chai *et al*., 2005).

For interrupting transmission at intermediate host, particularly snail population has not been succeeded (WHO, 2004). In fish infection control, irradiation of fish to prevent infectivity of metacercaria is tried for *C. sinensis* (Lee *et al*., 1989) and *O. viverrini* (Sornmani *et al*., 1993); however, feasibility of the method economically, and consumer acceptance appear to be obstacles to the use of this prevention method. While control program of OLFs, especially *O. viverrini*, has not been succeeded, population variation of parasites can be changed by host migration after monsoon flooding between different geographical origins of *O. viverrini* (WHO, 2004). Sriamporn *et al*., (2004) reported that *O. viverrini* infection ranges from 2% to 71% (mean 25%) in different districts within Khon Kaen Province. There is also variation in the incidence of CHCA in different communities in Thailand. However, there was no apparent correlation with the prevalence of *O. viverrini*. Although the role of genetic heterogeneity of infectivity in different hosts, transmission and associated disease is unknown, many studies indicated the cause of carcinogenesis in opisthorchiasis-associated CHCA and host factors which may play roles in pathogenesis such as degree of inflammatory response and genetic variability of antioxidant enzyme (Pinlaor *et al*., 2003; Honjo *et al*., 2005).

10. Diagnosis

Diagnosis is a very important issue for opisthorchiid liver flukes because of the difficulty of differentiating the egg of OLFs from those of Heterophyid intestinal flukes in human stool microscopic examinations. Normally, *O. viverrini* is often mixed infection with HIFs that may cause misdiagnosis and inaccurately estimating the prevalences of both *O. viverrini* and HIFs or by false negative in light infection and in biliary obstruction in which eggs can not be recovered in the feces (Lee, 1984; Ditrich *et al*., 1992; Ditrich et al., 1992; Waikagul, 1998; 2002; Chai and Lee, 1991; 2002; Sukoutason *et al*., 2001; Upatham and Viyanant, 2003).

Laboratory diagnosis of *O. viverrini* infection is usually based on recovery of egg from stool to diagnosis by morphological studies of egg worms such as Kato-thick smear, formalin-ethyl acetate concentration technique and direct simple smear. They are the simple techniques for stool examination diagnosis. It is suitable methods with

field studies at the endemic areas because the flukes' egg easily determined under a light microscope after smearing a faecal sample in a drop of normal saline solution and in cases of heavy infection when several thousand eggs per gram of feces are present, but it is low sensitive in cases of light infection (Pungpak, 1985; Sripa and Kaewkes, 2000; Wongratanacheewin *et al*., 2001; Waikagul, 2002). This method is widely used for separating worms and eggs being similar in size and shape under a light microscope. They are practically in distinguishable from the egg of many species of HIFs such as *H. taichui, H. yogokawai, H. pumilio, Stellantchasmus falatus, Centrocestus caninus, Metagonimus* sp, *Prosthodendrium molenkampi,* and *Plagiorchis harinasutai* (Ditrich *et al*., 1992; Radomyos *et al*., 1994; 1998).

Endeavors have been improved in the part to develop a sensitive and specific immunodiagnosis method, which would be more reliable in cases of light infection, in those with biliary obstruction and mixed intestinal parasitic infection (Sivisiha, 1986; Watthanakulpanich, 1997; Waikagul, 2000). Various immunodiagnostic methods are still studied widely by many investigators owing to their immunodiagnosis, the enzyme -liked immunoelectrotransfer blot (EITB) or immunoblot (IB) method and enzyme-linked immunosorbent assay (ELISA) systems.

Immunoelectrophoresis was previously studied by using crude extract of adult worm as an antigen, the sensitivity of the test was only 76% and patient sera occurred cross-reaction with other parasitic diseases including gnathostomiasis and schistosomiasis (Janechaiwat *et al*., 1980). By immunodiagnosis, profiles of the crude *O. viverrini* extract contain major proteins of 34-37, 43, 70 and 100 kDa. Cross-reaction is occurred with the sera of clonorchiasis at 100 kDa and the sera of other helminthes at 34-37, 70 and 100 kDa. The frequency and intensity of the immonoblot reaction are positively correlated with the intensity of the liver fluke infection (Choi *et al*., 2003). Wongratanacheewin and Sirisinha (1986) identified a protein with 89 kDa molecular weight of *O. viverrini* by radioimmumoprecipitation assay and found this protein to be a specific antigen of this parasite. This finding enlightens the possibility to develop a specifically serological assay for detecting *O. viverrini* antibodies in the infected host. However, sufficient qualities of this antigen and differentiation of active

reaction v.s. crude infection rat remain as major problems in diagnosis of opisthorchiasis.

Indirect ELISA for the detection of serum antibody in the patients is just as sensitive as the traditional microscopic procedure (Feldheim and Knoblooh, 1982; Thammapalered *et al*., 1988). Then, crude somatic antigen from *Bithynia fumiculata*-whole body is demonstrated as a good result as when *O. viverrini* antigen was used. The cut-off value for positive antigens, at 0.228, gives 80% sensitivity and 81.2% specificity, but cross-reactions are observed with sera from patient with paragonimiasis and strongyloidiasis (Watthanakulpanic *et al*., 1997).

Many studies in immunology have been developed for increasing the accuracy in detection and diagnosis of these groups. Various immunogilocal techniques have been used either by the detection of circulating antibody or copro-antigen (Upatham and Viyanant, 2003; Wongratanacheewin *et al*., 2003). However, the main problems of the detection of circulating antibody methods are 1) it is limited by the cross-reaction nature of antigens used (Akai *et al*., 1995; Sakolvare *et al*., 1997; Wongsarojet *et al*., 2001; Watthanakulpanich *et al* 1997) and 2) the positive result does not always indicate active infection of the positive (Viyanant *et al*., 1985; Thammapalerd et al.,1988; Akai *et al*.,1995; Upatham and Viyanant, 2003). Until now, mass indirect ELISA has been used for immunodiagnosis of opisthorchiasis under the different antigen preparations encountering with quantity of immune response of infected hosts. The antigens can be classified as excretion-secretion (ES) product of adult worms (Amornpunt,*et al*.,1991; Sirisinha *et al*,1991;1995; Sripa and Kaewkes,2000), crude adult worm, egg, and metacercaria (Srivatanakul *et al*., 1985; Wongratanacheewin *et al*., 1988; Akai *et al*., 1995; Sakolvaree *et al*.,1997), oval antigen (Wongsarojt *et al*., 2001; Sripa and Kaewkes, 2000),and snail antigen (Watthanapakulpanich *et al*.,1997; Waikagul *et al*., 2002).

11. Snail antigen

The relationship between *O. viverrini* adult worms and snail intermediate host, *Bithynai fumiculata, B. siamensis siamensis, B. s. goniomphalos*, has been confirmed

that antigen from parasite can be replaced by that of snail as an antigenic mimic material. The three-snail antigens produce two common precipitin bands against anti-*O. viverrini* serum by immunoelectrophoresis (Chanawong *et al*, 1990). By IgG-ELISA, four batches of crude antigens: 1) *O. viverrini* adult worms, 2) *B. fumiculata*-whole body, 3) *B. fumiculata*-head food, and 4) *B. fumiculata*-visceral mass, are comparatively analyzed and found that *B. fumiculata* antigen is mostly effective for diagnosis of *O. viverrini* infections. However, cross-reactions were occurred with sera from patients with paragonimiasis and strongyloidiasis (Watthanakulpanich *et al*., 1997).

Waikagul et al. (2002) evaluated that serodiagnosis of human opisthorchiasis using cocktail by Sephacryl S-200 HR gel filtration chromatography and electroeluted *Bithynia* snail antigens at the 53 kDa from polyacrylamide and the eluted antigen provided higher sensitivity and specificity of the diagnosis using indirect ELISA. However, cross-reactions with the eluted antigen were seen in case of hymenolepiasis and strongyloidiasis. The *B. s. goniomphalos* cocktail antigen used in this study gave a serodiagnostic sensitivity of 88.5% and specificity of 88%.

12. Iso-electric focusing

Iso-electric focusing (IEF) is an electrophotic method in which proteins are separated on the basic of their Iso-electric point (pI). At this point, the protein has an overall net charge of zero (Fig. 1) (Righetti, 1990). The pI is the pH at which a protein will not migrate in an electric field and is determined by the charged groups in the protein. The protein can move to positive, negative or zero (pH=pI) charge depending on their local pH, and for every protein. There is a specific pH at which its net charge is zero, but pH values usually fall in the rang of pH 3-10 with a great many having pI between pH 4 and pH 7 (Fig. 2) (Wilkins and Gooley, 1997). The proteins are positively charged in solution at pH values below their pI, proteins will move toward the cathode during electrophoresis while negatively charged above their pI is a protein will be move toward the anode during electrophoresis (Andrews, 1986; Wilkins and Gooley, 1997).

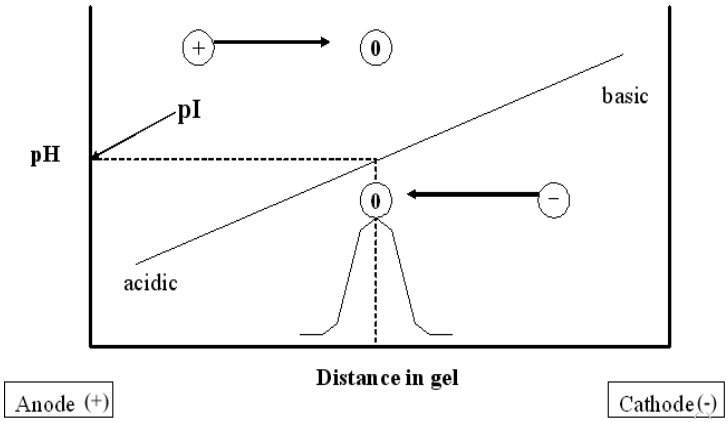

Figure 1: Iso-electric focusing theory, the motion of a protein undergoing iso-electric focusing is depicted (circles).

Reference: www. Bio-Rad.com

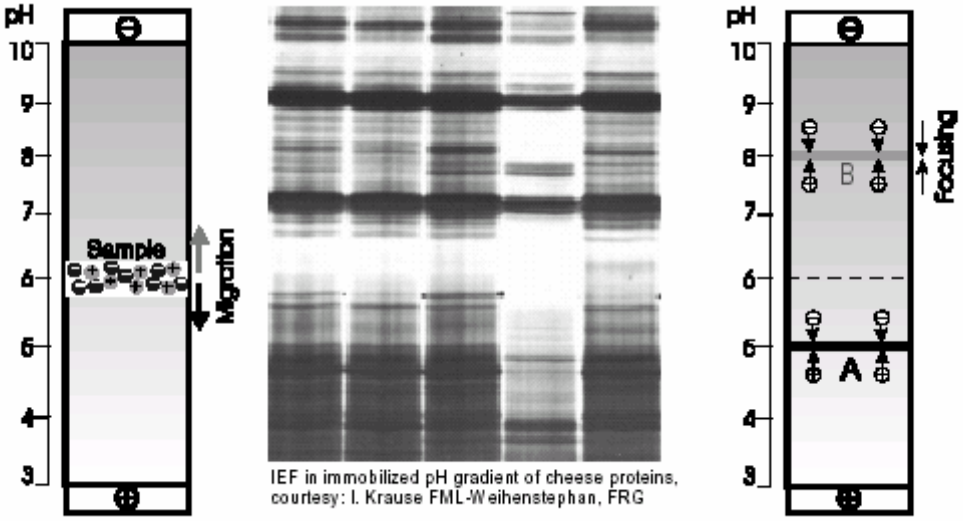

Figure2: Iso-electric focusing in mobilized pH gradient.

Reference: www. Bio-Rad.com

IEF is used as an alternative electrophoresis format complementing the widely used SDS-PAGE electrophoresis to solve problem about closely related protein have to be separated. This technique has been come crucial for the development of proteolysis. Examples are the differentiation of proteins isoform or enantiomers that problems have been successfully solved by iso-electric focusing (Glukhovskiy *et al*., 1999). To develop differentiation of separating proteins under the same principle of IEF toward appropriate technique for separate proteins. The different application

requires different IEF method such as IEF in ultrodex gels, IEF with Ampholine and Pharmlyte, IEF in Agarose gels, IEF in polyacrylamide gels, IEF in rehydrated polyacrylamide gels, IEF in immobilized pH Gradient, IEF in gel strips with immobilized pH Gradients (Righetti *et al.*, 1983; Westermeier, 1993; Righetti, 1993; Wurster, 1998).

Proteins of homogenized adults *O. viverrini* are separated by IEF an relative abundance of about 47% only one or two protein components appears to be unique for *O. viverrini* among helminths and a double band of MW 18 and 19 kDa as by for the most prominent component (Rupple *et al*., 1985). Viyanant and Upatham (1993) evaluated that isoenzyme patterns of adult *Schistosoma malaysian, S. mekongi*, and *S. japonicum* strains were analyzed by IEF in polyacrylamide gels from enzyme patterns obtained from *S. malayanum* homogenate that was differed from *S. mekongi*, and *S. japonicum* strains. The IEF in liquid phase method is shown to be more effective of protein detection that is below 40 kDa (Wall *et al*., 2001), and it is an alternative for separating complex protein mixtures (Karty *et al*., 2007). This technique is used for protein separation between two species of liver fluke, *Fasciola hepatica* and *F. gigantica*. It is demonstrated that the banding patterns can be used to distinguish 1 species from the other. The protein is simple, reproducible and good resolution (Lee *et al*., 1993).

13. Advantages for the IEF method

The advantages of IEF method against the other purifications method include;

1). Optimal resolution in other electrophoretic systems requires application of sample as a narrow zone

2). IEF is not so crucial, even large sample volumes do not lower resolution.

3). Sample concentration upon focusing also results in lower detection limit compared with other electrophoresis techniques.

4). This technique does not require denaturation of protein, thus any kind of subsequent investigation, such as actively staining or antibody detection, is not hindered.

5). The protein becomes lightly concentrated at their pI. This results in a high sensitivity for detection. Small charge difference can be differentiated.

When it is compared to alternative methods; IEF offers the following advantages as efficient, expressive, economic, easy, fast, and density (Rigetti, 1989; Andrews, 1986; Gluhovskiy *et al*., 1999; Link, 1999; Corthals *et al*., 2000).

CHAPTER IV
MATERIALS AND METHODS

1. Serum samples

1.1 Samples collection

Serum samples used in this study were obtained from the Department of Helminthology, Faculty of Tropical Medicine, Mahidol University. Each serum sample was aliquot stored at -70 ° c until used.

Serum samples were confirmed by many methods for diagnosis of parasitic infections, which could be divided into three groups.

Group A: sera of opisthorchiasis

Fifty serum samples of opisthorchiasis were obtained from patients who had presented worms in stool samples. Direct smear and Kato's thick smear were firstly used for finding eggs of *O. viverrini* or similarity and then patients would be given treatment. Their stools were examined for adult worms. Then, serum samples were collected from those patients (table/Table 1).

Group B: sera of other parasitic infections

The heterologous sera that were composed of 24 kinds of helminthic infections and protozoa infections were confirmed by many diagnostic methods depending on parasitic infections (Table 1).

Group C: sera of healthy individuals

Thirty-five healthy sera control were obtained from 513 volunteers whose stool samples were negative by simple smear technique (Table 1). All, 513 serum samples, were confirmed again by immune testing because fecal screening tests are not enough to interpret purification of serum samples. It is necessary to allocate serum negative controls by using simple indirect-ELISA for low antibody detection. ELISA tests were used ten kinds of antigens, crude and partially purified, as following parasites; *Gnasthostoma spinigerrum* larvea, *Angiostrongylus cantonensis* adult worms, *Strongyloides stercolaris* infective larvae, *Dirofilaria immitis* adult worms, *Toxocara*

canis adult worms, *Trichinella spiralis* muscle larvae, *Taenia solium* metacestodes, fluid of hydrated cyst, *Paragonimus heterotremus* adult worms, and *Fasciola gigantiga* adult worms. The low-OD values had previously been determined by simple statistic analysis.

The study protocol was approved by the Scientific-Ethical committee of Mahidol University.

Table 1. Demonstration on serum samples of opisthorchiasis (A), heterologous infections (B), and healthy controls (C) with numbers, positive results of diagnostic techniques

Group	Diseases	Number	Diagnostic detections
A	Opisthorchiasis (Ov)	50	Worms
	Total	**50**	
B	Gnathostomiasis (Gn)	12	Worm, immunoblot
	Strongyloidiasis (Ss)	10	Larvae
	Hookworm infection (Hw)	10	Larvae
	Trichinellosis (Tcn)	10	Larvae, immunoblot
	Capillariasis (Cp)	3	Eggs, larvae, adult worm
	Toxocariasis (Tx)	10	Immunoblot
	Angiostrongyliasis (Ang)	10	Worm, immunoblot
	Ascariasis (As)	10	Eggs, worms
	Trichuriasis (Tt)	10	Eggs, worms
	Bancroftian filariasis (Wb)	10	Microfilariae
	Brugian filariasis (Bm)	10	Microfilariae
	Dirofilariasis (Df)	1	Worm
	Neurocysticercosis (Ncc)	10	Cysts, immunoblot
	Sparganosis (Sp)	3	sparganum

Group	Diseases	Number	Diagnostic detections
	Taeniasis (Ta)	10	Eggs, segments
	Echinococcosis (Ecc)	10	Protoscolices
	Hymenolepiasis nana (Hm)	4	Eggs, worms
	Paragonimiasis heterotremus (Ph)	10	Eggs, worms
	Schistosomiasis (Sc)	4	Eggs, serodiagnosis
	Fascioliasis (Fas)	4	Eggs, immunoblot
	Haplorchiasis (Ha)	10	Worms
	Creeping eruption (Cr)	3	Symptoms, negative for strongyloidiasis and gnathostomiasis
	Entamoebiasis (En)	3	Cysts
	Giardiasis (Gd)	4	Cysts
	Blastocystis homonis infection (Bh)	4	Cysts
	Malaria (Ma)	5	Blood stages
	Toxoplasmosis (Txp)	3	Immuno-diagnosis
	Total	**196**	
C	Healthy serum (Hs)	35	Stool examinations, ELISA
	Total	**279**	

1.2. Calculation of sample size

The population size in this study was followed the number of samples in statistical criteria 'Estimating a population proportion: One-group study' following the formula:

$$n = \frac{Z^2_{1-\alpha/2} p(1-p)}{d^2}$$

n = the sample size

Z = the standard normal deviate (1.96 for a 95% confidence interval)

p = the estimated positive rate (p = 0.8 = 80%)

(From the previous study, it was found the rate is unlikely to exceed 20%)

d = 0.05 = 5% the level of absolute precision, or sampling error, or one half the width of the confidence interval.

Thus

at 95% confidence interval; $Z_{0.05,\alpha} = 1.96$

$$n = \frac{(1.96)^2 (0.2)(0.8)}{0.05^2} \approx 246$$

Therefore, at least 279 persons were determined and all fifty sera of opisthorchiasis were included in the study.

2. Preparation of purified *Bithynia siamensis goniomphalos* snail intermediate host

2.1. *B. s. goniomphalos* snail

The living *B. s. goniomphalos* snails, which were used in the study had been previously bred and cultured in the laboratory by the Applied Malacology

Center, the Department of Social and Environment Medicine, Faculty of Tropical Medicine, Mahidol University.

2.2. Screening for natural parasitic infection

Although these snails were the laboratory breeding, all of snails were still needed to ensure that they were free from any natural parasitic infection. This minimized undesirable effect when they were used in the experiments. The examinations were already done for shedding of developmental stages of a parasite and free-living trematode and crushing technique, which was modified for this examination as the following detail. Each snail was placed in 3.5 cm diameter plastic cup containing 10 ml of dechlorinated tap water and kept under fluorescent light for 8-10 hrs. The presence of cercariae in water was observed using stereomicroscope. The positive infection with cercariae was discarded. Only uninfected snails were gently crushed and all pieces of their shells were removed. The body of snails was examined for any developmental stage of parasites (sporocyst, redia, or cericaria) under a stereomicroscope. Only bodies of uninfected snails were collected. About 150-200 snails were used for preparing the antigens.

3. Preparations of antigens

3.1. Extraction of snail crude antigen

The uninfected *B. s. goniomphalos* snail bodies were extracted to be crude antigen. Every 50 snails was ground by a glass pestle and mortar in small amount of sterile distilled water (DW) and alumina on ice. The homogenated protein was sonicated by sonicator® model 2020 at 20 times for an interval of 1 min with a probe No 418, magnification No.4 on ice and then centrifuged at 20,000 rpm for 60 min at 4°C. The supernatant was filtered trough glass wool to eliminate mucous of snails and collected in small plastic tubes and then stored at -70°C. The supernatant was determined for its protein content.

3.2. Iso-electric focusing-fractionated antigens (IEFA)

The crude antigen (110 mg) was fractionated by preparative IEF using the Rotofor cell (Bio-Rad) according to Ito *et al* (1999) and the Bio-Rad's protocol. Briefly, the antigen was desalted by dialysis and then mixed with 40 ml Tris buffer,

pH 8.8 and 2 ml ampholyte [40% (w/v), pH 3-10] for making 2%ampholyte. The samples were loaded into the Rotofor cell using a 1-1/2 inch 19-gauge needle. During filling, air bubbles could become trapped in the electrolyte chamber thus the bubble must be removed for avoiding discontinuity of electrical field. The electrolytes for the anode and cathode were 0.1M H_3PO_4 and 0.1M NaOH, respectively. The samples were subjected to IEF at a constant power of 12 W at 4°C for 4 hrs. The test tube rack with 20 culture tubes in serial numbers was placed into the harvest box. A vacuum source was connected to the vacuum port on the harvest box and turned on the vacuum pump. The 20 fractions were simultaneously aspirated from the cell and delivered to the fraction tubes. All fractionated were measured their pH and then dialyzed against DW.

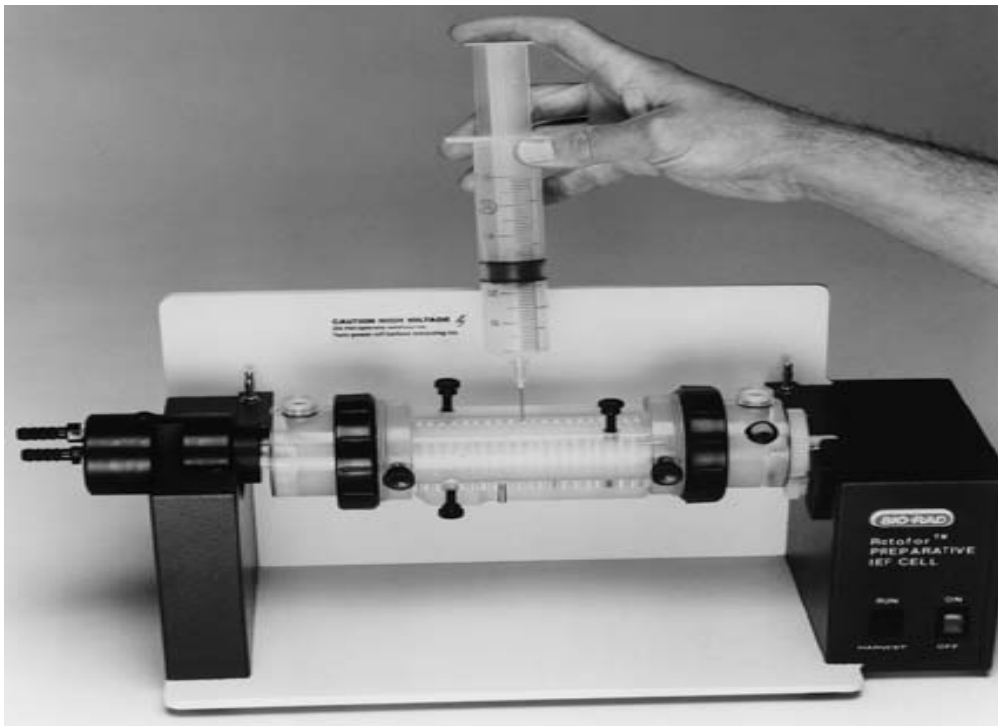

Figure 3: Loading the samples.

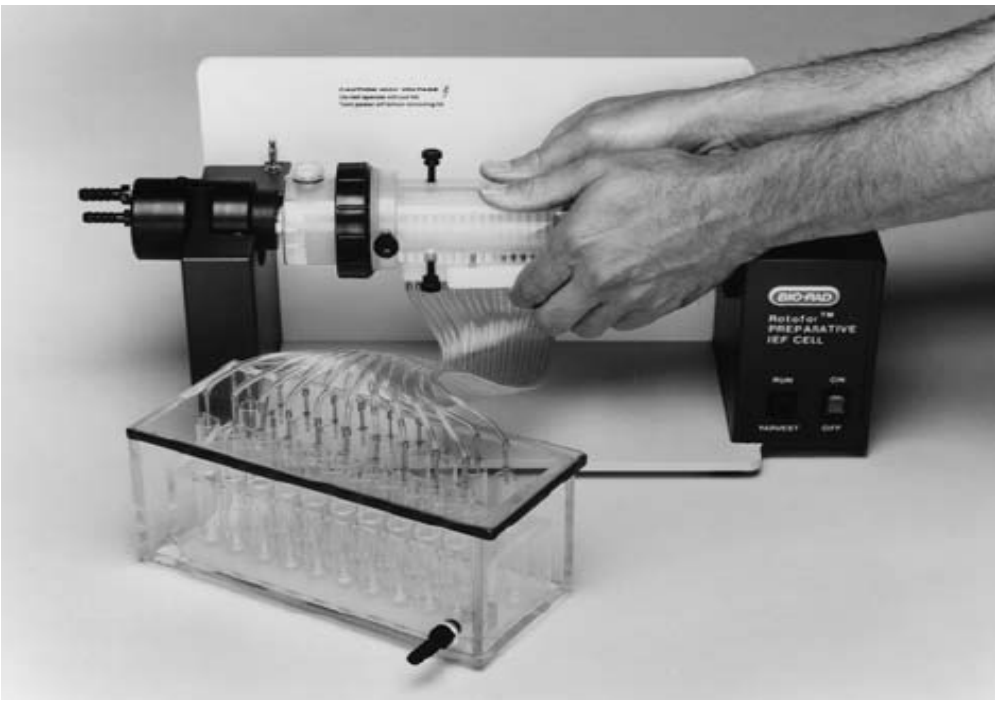

Figure 4: Harvesting samples after focusing is complete

4. Protein determination

Protein determinations of all antigens were calculated by a protocol of Comassie® plus protein assay reagent kit (PIERCE), using bovine serum albumin (BSA) as a standard. The serial dilutions were performed with BSA at 20, 10, 5, 2.5, and 1.25 μg/ml, and the serial dilutions of all antigens were prepared with distilled water at 1:50, 1:100, 1:200 and 1:400 respectively. A mixture occurred for the activity in a microtiter plate between 150 μl of Comassie® plus protein with 150 μl of the serial dilutions of BSA and with 150 μl the serial dilutions of all antigens, ELISA was employed in evaluation of the antigens. Absorbance values were measured at 595 nm. A protein concentration of antigen was automatically determined by Microsoft® office Excel 2003 program against a BSA standard curve.

5. Sodium dodecyl sulphate-polyacrylamide gradient gel electrophoresis (SDS-PAGGE)

The crude and fractionated antigens were analyzed by SDS-PAGGE consisting of 5% stacking gel and 10%, 12%, 15%, and 17% gradient gel following a protocol of

ATTO Laboratories, Japan, with some modifications. The gradient gel was prepared in a vertical slab gel apparatus by hand manipulation, overlaid with DW and allowed to polymerize for 1 hr at room temperature. After DW was removed, a stacking gel solution was prepared and poured on the separating gel and inserted a multi-well comb. Polymerization of gel was allowed at least 1 hr. A volume of 20 individually fractionated antigens and the same concentration of crude antigen were treated with an equal volume of sample buffer (1.5X; 0.25 M Tris-HCl, pH 6.8, 1.75% SDS, 3.75% mercaptoethanol, 7.5% glycerol, and 0.008% bromophenol blue) then heated at 100°C for 3 min in a heat block (Accublock^{TM} , Labnet). Each well of stacking gel was loaded with 10 μl of the treated complexes including a well of low molecular weight protein standard markers (Bio-Rad). The separation of all samples was done by a constant current at 20 mA until the tracking dye reached the bottom of the gradient gel. The protein bands in a gel were stained with Coomassie brilliant blue for 1 hr. Excess stain was removed by placing the gel in destaining solution with several changes until the background of the gel was clear. Molecular weight determination of unknown protein bands were calculated by comparing their relative mobility against a standard curve making from those of protein standard markers.

6. Selection of antigens for full scale ELISA

Due to several fractions of snail antigen, a system of antigen selection was determined by using ELISA-application of Voller *et al*. (1979) and Dekumyoy *et al*. (1998) with minor modification. The selection was done by comparison of OD-ELISA ratios between positive pooled serum and negative pooled serum using each fractionated antigen. Each positive and negative serum samples were obtained from seven randomly chosen samples. Antigenic fraction was diluted at 1 μg/ml reacted with 1:100 dilution of both pool sera, and then followed with 1:2,000 dilution of conjugate (total IgG) and Antigenic fraction was diluted at 2 μg/ml reacted with 1:200 dilution of the pool sera, and reacted with 1:1,000 dilution of conjugate (IgG subclass, 1-4). Each conjugate, all OD-ratios were statistically analyzed and high OD-ratios were limited in a range of mean plus high SD would be selected for further full scale ELISA, which corresponded to an antigen used.

7. Enzyme-linked immunosorbant assay (ELISA)

Indirect ELISA was employed in evaluation of the antigens against sera from patients infected with *O. viverrini*, heterologous infections and healthy controls. ELISA was performed in microtiter plates as described by Dekumyoy et al., (1998) with minor modification. Checkerboard titrations of an antigen against pooled positive and negative sera at varioue conjugate dilutions were performed in order to establish the optimum conditions for the tests.

An antigen was diluted with 0.05M carbonate-bicarbonate buffer pH 9.6 to the concentration of 0.5, 1, 2, 4 µg/ml. Fifty microliters of antigen dilutions were added to each well of microtiter plate (Nunc, Denmark). The plate was incubated at 37^{o}C for 1 hr and followed by an overnight at 4^{o}C. Then, the unabsorbed antigens were removed from the plate by washing with 0.1 M PBS, pH 7.4 containing 0.05% Tween 20 (PBS-T) by a microshaker, 3 times, 1 min each. The surface's wells with bound antigen were blocked with 75 µl of 0.5% BSA in PBS, pH 7.4-0.02% sodium azide, incubated for 1 hr at 37^{o}C, and washed as above. The pool sera were serially diluted at 1:100, 1:200, 1:400, and 1:800 with diluent (washing solution containing 0.02% NaN_3 – 0.2% bromphenol blue) and 50 µl of each dilution were put in a concentration of antigen by duplicate well. The reactions were done at 37^{o}C for 1.5 hr and then washed for 5 times. The immune complexes combined with 50 µl of a serial dilution of each conjugate (horseradish peroxidase-labeled anti-human IgG, and IgG1-4, Southern Biotech), incubated at 37^{o}C for 1.5 hr and washed as above. The reactions were visualized with 50 µl of substrate solution [ABTS; 2, 2-azino-di-(3-ethyl-benzthiazoline sulfonate)] after a 30-min incubation. Absorbance values were measured at 405 nm after the addition of 1% SDS to stop reaction.

The titration curves were plotted from the absorbance values of the serial dilutions of the sera and conjugates against various concentrations of the antigens.

After the checkerboard titration, the optimal concentration of various antigen preparations, reference sera and conjugate were performed for further teats with sera of opisthorchiasis, heterologous infections and healthy controls. Sensitivity and specificity, Positive-Negative predictive values were evaluated.

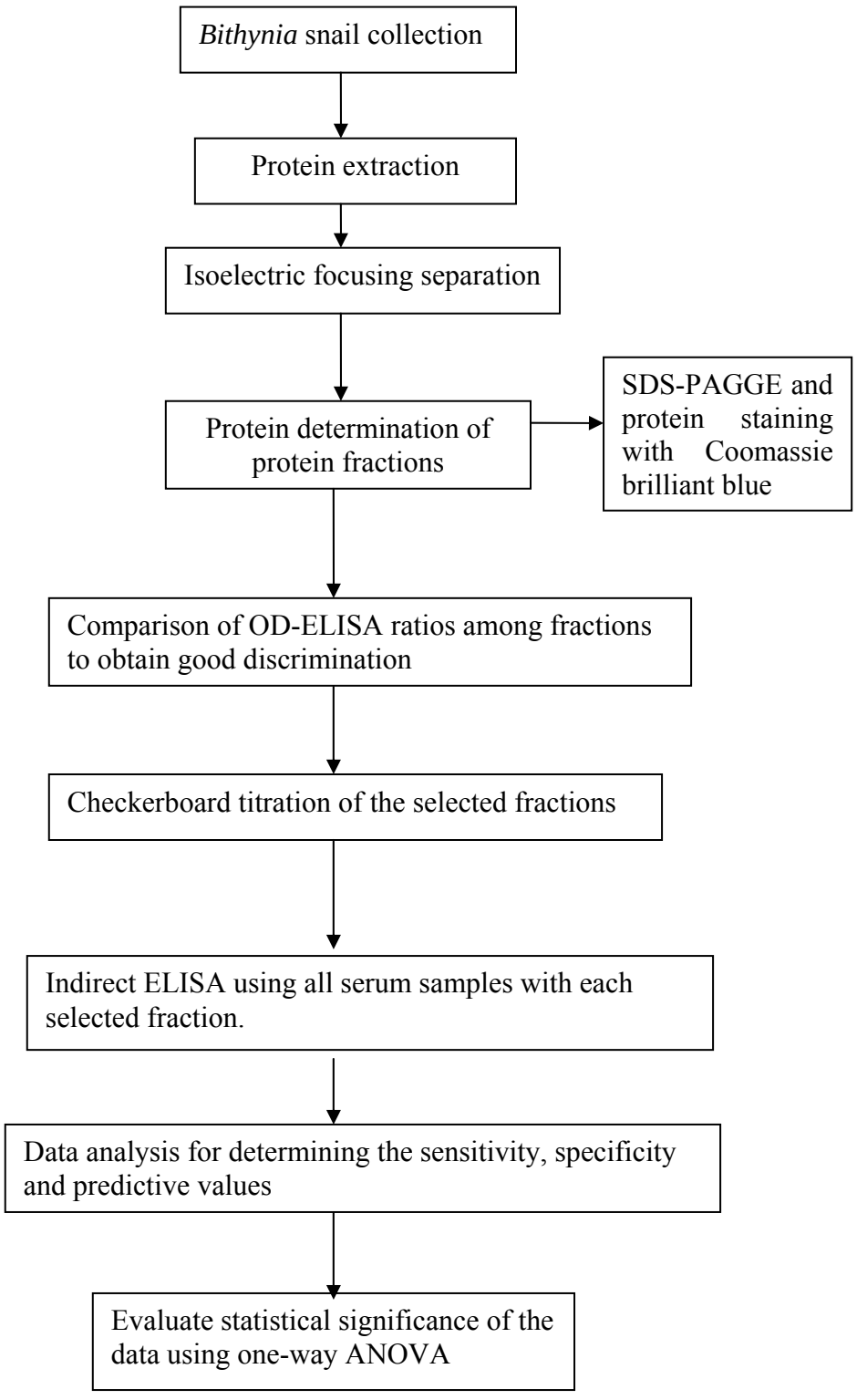

Fig. 5. conceptual frameworks in this study

8. Data analysis

8.1. Sensitivity, specificity and predictive values

The diagnosis accuracies of the test, sensitivity, specificity and predictive values, were determined using the method of Galen (1979). Mean ($\overline{X}$), standard deviations (SD) and ranges of optical densities (OD) were used to analyse in ELISA. The ELISA has a set of characteristics that reflect the information expected in patients with and without diseases. These characteristics can be easily displayed by a simply binary table often referred of as a "2 by 2" table, as shown below:

		Disease	
		Present	**Absent**
Positive		TP	FP
Negative		FN	TN

TP = Number of patients with opisthorchiasis in whom the test is positive (true positive).

FP = Number of patients without opisthorchiasis in whom that test is positive (false positive).

FN = Number of patients with opisthorchiasis in whom the test is negative (false negative).

TN = Number of patients without opisthorchiasis in whom the test is negative (true negative).

8.1.1. Sensitivity

The sensitivity of a test is the probability that the test procedure or result will be positive when the opisthorchiasis is present. It will be determined by identifying the proportion of patients with opisthorchiasis in whom the test is positive.

$$\textbf{Sensitivity} = \frac{\text{True positive (TP)}}{\text{True positive (TP)} + \text{False negative (FN)}} \times 100$$

8.1.2. Specificity

The specificity is probability that the test will be negative when opisthorchiasis is not present. It will be determined by identifying the proportion of patients without opisthorchiasis in whom the test is negative.

$$\textbf{Specificity} = \frac{\text{True negative (TN)}}{\text{True negative (TN) + False positive (FP)}} \times 100$$

8.1.3. Positive predictive value

The positive predictive value is the probability that opisthorchiasis is present when the test is positive.

$$\textbf{Positive predictive value} = \frac{\text{True positive (TP)}}{\text{True positive (TP) + False positive (FP)}} \times 100$$

8.1.4. Negative predictive value

The negative predictive value is probability that opisthorchiasis is absent when the test is negative.

$$\textbf{Negative predictive value} = \frac{\text{True negative (TN)}}{\text{True negative (TN) + False negative (FN)}} \times 100$$

8.2. Statistical analysis

The curve analyses were performed from OD-ELISA values, which were used to select a cut-off OD value for differentiating among three groups of sera human (group A: sera of opisthorchiasis, group B: sera of other parasitic infections, and group C: sera of healthy individuals) by mean and SD including, the sensitivity, specificity and predictive values from OD-ELISA were calculated by SPSS version 13.0 program

windows to analyze the association between groups of fractioned antigen and total IgG, IgG1, IgG2, IgG3 and IGg4. While SPSS program windows was also used to evaluate for significant statistical comparing in each experimental group by using one-way ANOVA.

The ANOVA test was used to evaluate statistical significance at the 5% level. The median values among dependent groups were performed using the Fisher's Least-Significant Difference (LST).

CHAPTER V
RESULTS

1. Preparation of crude *Bithynia siamensis goniomphalos* snail

In the previous study, the crude somatic *B. s. goniomphalos* snail antigen contained protein content 18 mg per ml.

2. Preparation of Iso-electricfocusing-fractionated antigens (IEFAg)

The crude antigen from *Bithynia* snail (110 mg) was fractionated by preparative IEF using the Rotofor cell. Twenty non charge-fractionated antigens were obtained following the gradient pH (Table 2).

Table. 2: Demonstration of protein concentration and pH of following fractions separated by Iso-electric focusing technique

Fraction No.	Protein Concentration		pH
	µg/ml	Total Protein (mg)	
Iso-F Ag 1	0.32	0.74	3.7
Iso-F Ag 2	0.36	0.76	4.1
Iso-F Ag 3	0.70	1.68	4.9
Iso-F Ag 4	0.75	1.80	5.3
Iso-F Ag 5	0.96	2.30	6.3
Iso-F Ag 6	0.89	2.05	6.5
Iso-F Ag 7	1.06	2.44	7.0
Iso-F Ag 8	0.69	1.66	7.1
Iso-F Ag 9	0.55	1.32	7.2
Iso-F Ag 10	0.51	1.17	7.3
Iso-F Ag 11	0.67	1.61	7.4
Iso-F Ag 12	0.79	1.90	7.5
Iso-F Ag 13	0.64	1.54	7.6
Iso-F Ag 14	0.82	1.97	7.7
Iso-F Ag 15	0.80	1.92	8.0
Iso-F Ag 16	0.72	1.73	8.2
Iso-F Ag 17	0.65	1.49	8.4
Iso-F Ag 18	0.60	1.38	9.0
Iso-F Ag 19	0.55	1.55	9.3
Iso-F Ag 20	0.30	0.72	9.6

The contents of proteins in all iso-fractioned antigens varied in a range of 0.30-1.06 mg/ml, which corresponded to a ranging protein content, 0.72-2.44 mg. It contained a total protein of 31.73 mg (28.84%). The highest protein content was 2.44 mg in fraction 7, pH 7.

3. Sodium dodecyl sulphate-polyacrylamide gradient gel electrophoresis (SDS-PAGGE)

Twenty fractioned proteins were separated according to their native charges in the gradient pH 3-10 using Rotofor cell (Bio-Rad). Then, SDS-PAGGE separated the characteristic snail extracts from the 20 iso-fractions and detected by Coomassie brilliant blue. The protein contents were realizable to demonstration by applying a constant volume (15 μl) of each fraction. It was found that somatic *B. s. goniomphalos* snail extract in all fractions contained different protein contents. Majority of banding pattern was seen as strong and defined bands in some fractions and their molecular mass similarity were between 97.4-21.5 kDa (standard markers) (Fig. 6). However, fractions 1, 2, 9, 10, 15, 16, 17, 18, 19, and 20 gave very weak detection of proteins, especially small molecular weights of fractions 15, 16, 17, 18, 19, and 20 in a range of 35.5-22 kDa_(standard markers). Using the iso-electric focusing technique, the nature of crude somatic *B. s. goniomphalos* snail extract carried several protein contents at pH 7, which corresponded to the fraction 7 (Table 2). In each separation, crude somatic proteins were also included for banding comparison.

When detection of 20 iso-fractioned proteins by the same concentration (10 μg), all fractions revealed high degree of similarity of protein bands and several defined bands were seen, especially 50.3, 44.5, 40.6, 37.2, 32.3, 29.2, and 26.8 kDa. The molecular mass were mainly in a range of 13.5 to 90 kDa and defined bands could be seen at a range of > 62.5 – 26.8 kDa and other bands were from 13.5 to 90 kDa as 53.05, 50.3, 46.62, 40.62, 37.23, 31.87, 29.25, 28.62, 26.84, and 23.87, respectively) (Fig. 7).

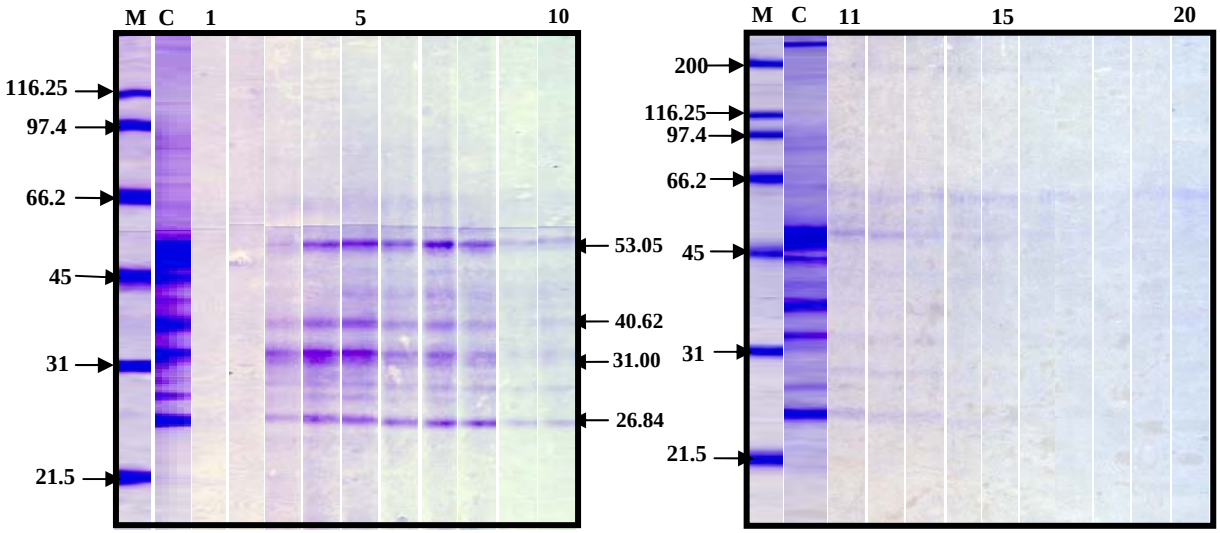

Figure 6: SDS-PAGGE separated iso-fractioned snail proteins (lane 1-20) by the same volume, and crude *Bithynia* snail extract (lane C), which stained with Coomassie brilliant blue. Molecular weight standards (M) were paralleled in a separation.

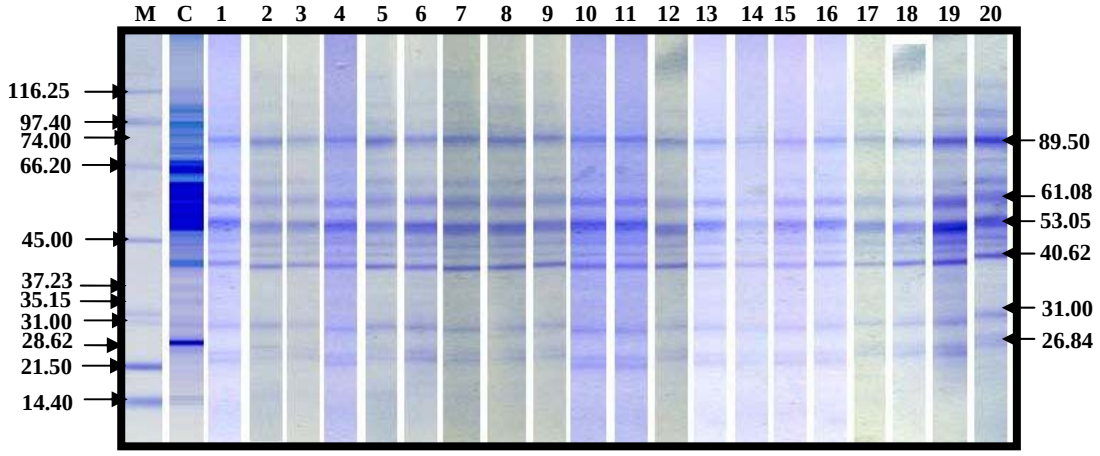

Figure 7: SDS-PAGGE separated iso-fractioned snail proteins (lane 1-20) by the same concentration, and crude *Bithynia* snail extract (lane C) and which stained with Coomassie brilliant blue. Molecular weight standards (M) were paralleled in a separation.

4. Selection of iso-antigens by ratio-ELISA

A selection of the 20 Iso-fractioned snail antigens was determined by ELISA-ratio comparison. Using the same antigen concentration (1µg/ml), 1:200 dilution of both pool sera (specific antibodies to *O. viverrini* and healthy control serum) and then followed with 1:2,000 dilution of conjugate (total IgG). It was found that antibody to *O. viverrini* showed quite high ODs-ELISA in range of 0.365 – 0.955 and pooled healthy control serum giving 0.106 – 0.274. The fractionated antigens were selected based on a high ELISA ratio as shown in Fig. 8 and Table 3.

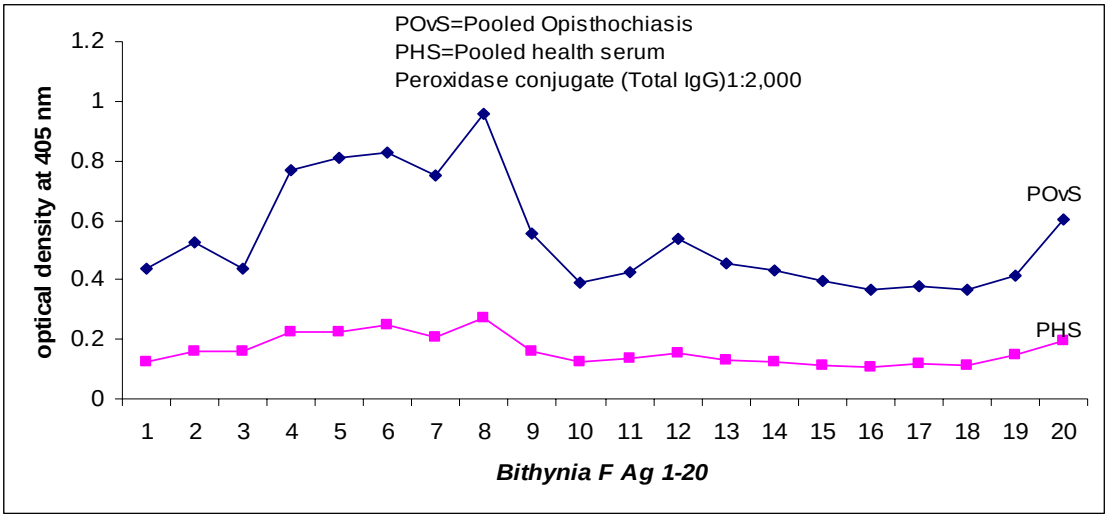

Figure 8: Demonstration of titration curves between antibody *O. viverrini* serum (POvS= pooled Opisthorchiasis serum) and healthy control serum (PHS= pooled health serum) against Iso-fractioned snail antigen (Iso-SAgF) 1-20 and encountered with peroxides conjugate (total IgG) at 1:2,000.

The 20 iso-fractioned snail antigens were also determined by the same manner, antigen concentration (2µg/ml), 1:200 dilution of both pool sera (specific antibodies to *O. viverrini* and healthy control serum) and then followed with 1:2,000 dilution of conjugates (IgG1-4). The ODs-ELISA of IgG subclasses were individually demonstrated in comparison between opisthorchiasis serum and healthy control serum in Figures 9-12. The fractionated antigens were selected based on a high ELISA ratio as shown in Table 3.

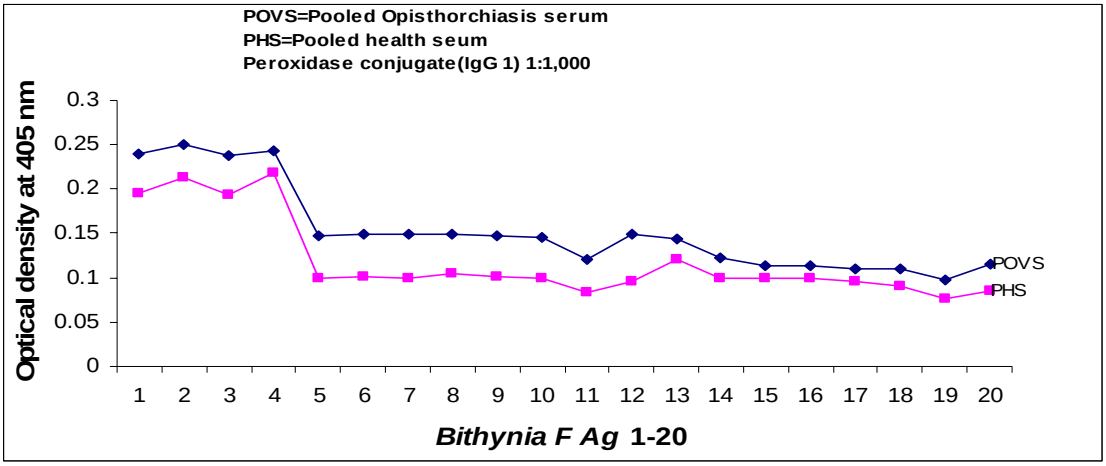

Figure 9: Demonstration of titration curves between antibody *O. viverrini* serum (POvS= pooled Opisthorchiasis serum) and healthy control serum (PHS= pooled health serum) against Iso-fractioned snail antigen (Iso-SAgF) 1-20 and encountered with peroxides conjugate (IgG 1) at1:1,000

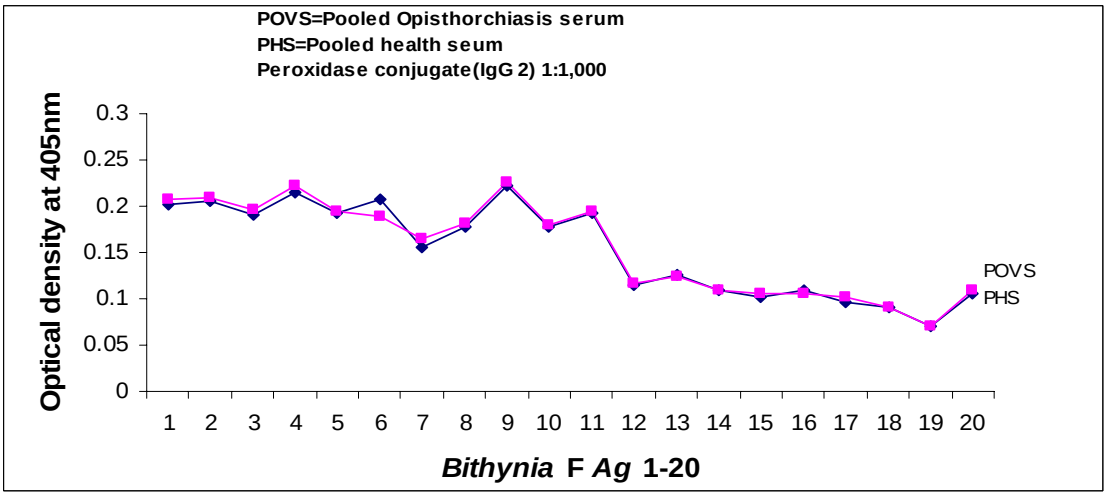

Figure 10: Demonstration of titration curves between antibody *O. viverrini* serum (POvS= pooled Opisthorchiasis serum) and negative serum (PHS= pooled health serum) against Iso-fractioned snail antigen (Iso-SAgF) 1-20 and encountered with peroxides conjugate (IgG 2) at 1:1,000.

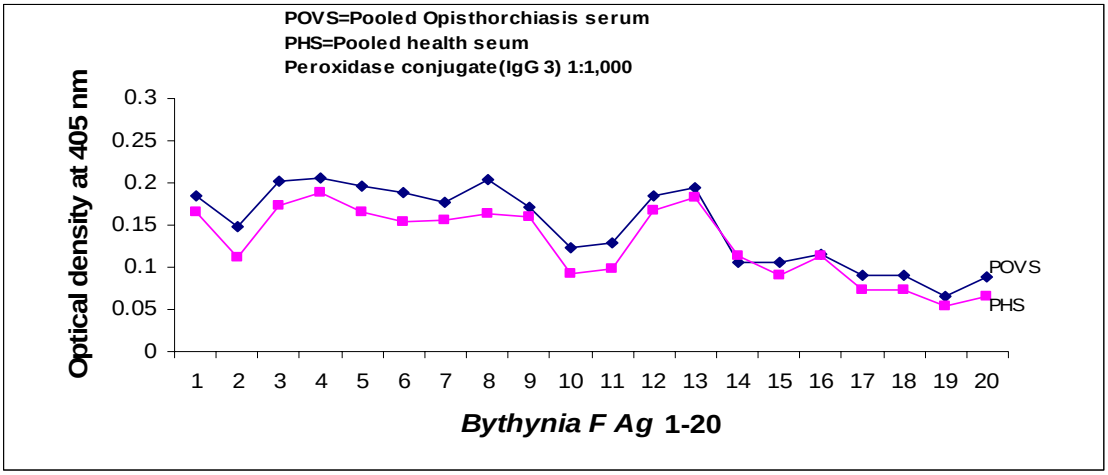

Figure 11: Demonstration of titration curves between antibody *O. viverrini* serum (POvS= pooled Opisthorchiasis serum) and negative serum (PHS= pooled health serum) against Iso-fractioned snail antigen (Iso-SAgF) 1-20 and encountered with peroxides conjugate (IgG3) at 1:1,000.

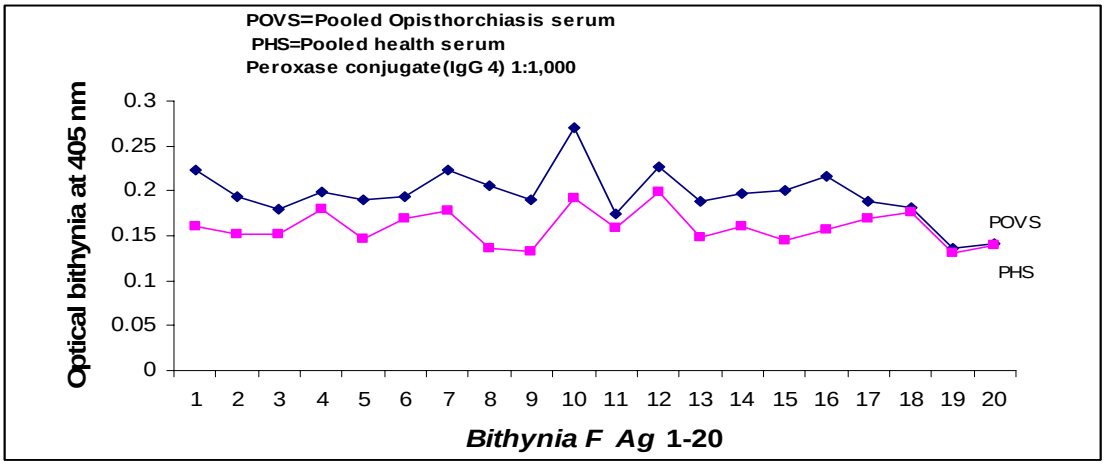

Figure 12: Demonstration of titration curves between antibody *O. viverrini* serum (POvS= pooled Opisthorchiasis serum) and negative serum (PHS= pooled health serum) against Iso-fractioned snail antigen (Iso-SAgF) 1-20 and encountered with peroxides conjugate (IgG4) at 1:1,000.

The OD-ELISA values gave differentiation in each fractioned antigens and each subclass of IgG, when they were reacted with pool positive and pool negative. It is depending on specific reaction in the treatment groups. In this study, we selected candidate antigen by the highest OD-ELISA ratios based on IgGs. They were composed of FAg 7, FAg7, FAg 6, FAg 2, and FAg 10, which reacted with total IgG,

IgG1, IgG2, IgG3, and IgG4, respectively. The selected antigens and the highest ratio of ELISA values indicated in Table 3.

Table: 3. Five candidate antigens were selected for full scale ELISA following antibody isotypes and the highest ratio.

The selected antigens	Antibody isotype	Highest ELISA ratio
Iso-FAg 7	Total IgG	3.6
Iso-FAg 7	IgG 1	0.049
Iso-FAg 6	IgG 2	0.018
Iso-FAg 2	IgG 3	0.032
Iso-FAg 10	IgG 4	0.047

5. Checkerboard titration for full scale ELISA

5.1 Determination of the antigen, reference serum and the conjugate (Total IgG)

In order to optimize the concentration of *Bithynia* snail antigen, iso- F 7, the dilutions of pool- positive and pool-negative controls and HRP-conjugated rabbit anti-human IgG (total IgG) were used for the indirect ELISA. The conditions were applied in the reactions as followed: the antigen dilutions, 0.5, 1, 2, 4 and 8 µg/ ml, the pool reference sera serially diluted from 1:100, 1:200, 1:400 and 1:800 and then using peroxidase conjugate (totol IgG) at 1:2,000, 1:4,000, and 1:8,000. Their ELISA conditions were determined by checkerboard titrations in Figs. 13- 15.

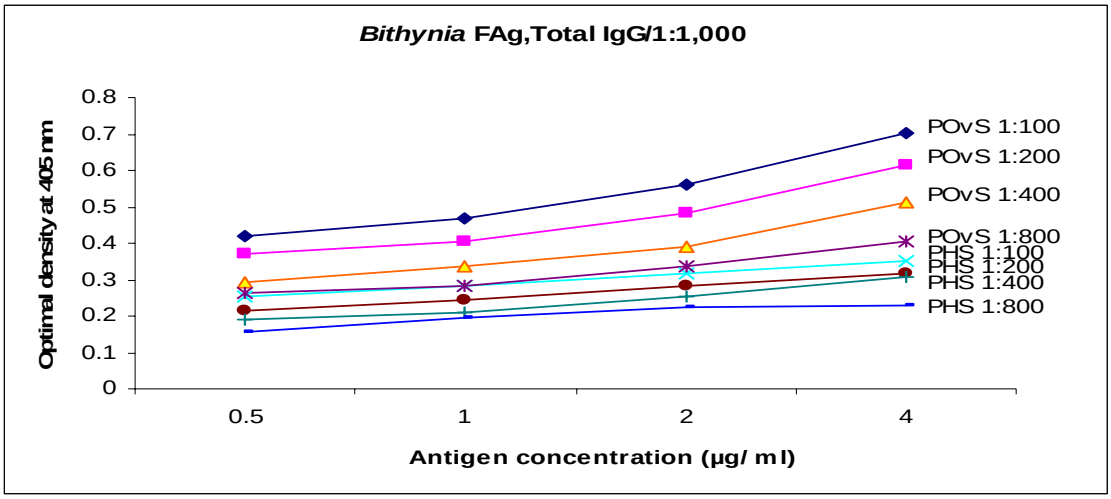

Figure 13: Checkerboard titration curves from the indirect ELISA for determination of the optimal concentration of Iso- FAg 7 and sera (POvS = pooled Opisthorchiasis serum and PHS = pooled health serum) by using peroxidase conjugate (total IgG) at 1:1,000

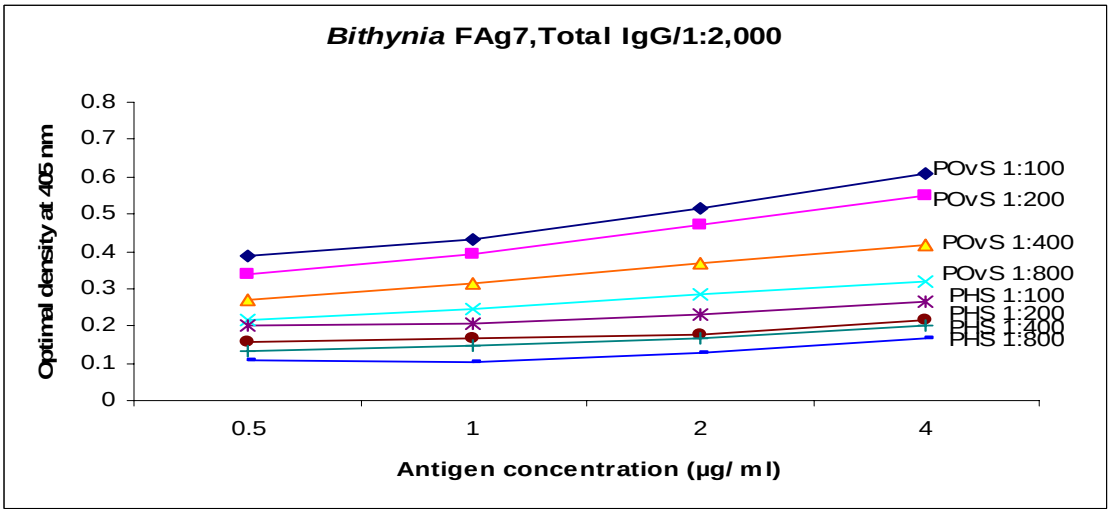

Figure 14: Checkerboard titration curves from the indirect ELISA for determination of the optimal concentration of Iso- FAg 7 and sera (POvS = pooled Opisthorchiasis serum and PHS = pooled health serum) by using peroxidase conjugate (total IgG) at 1:2,000

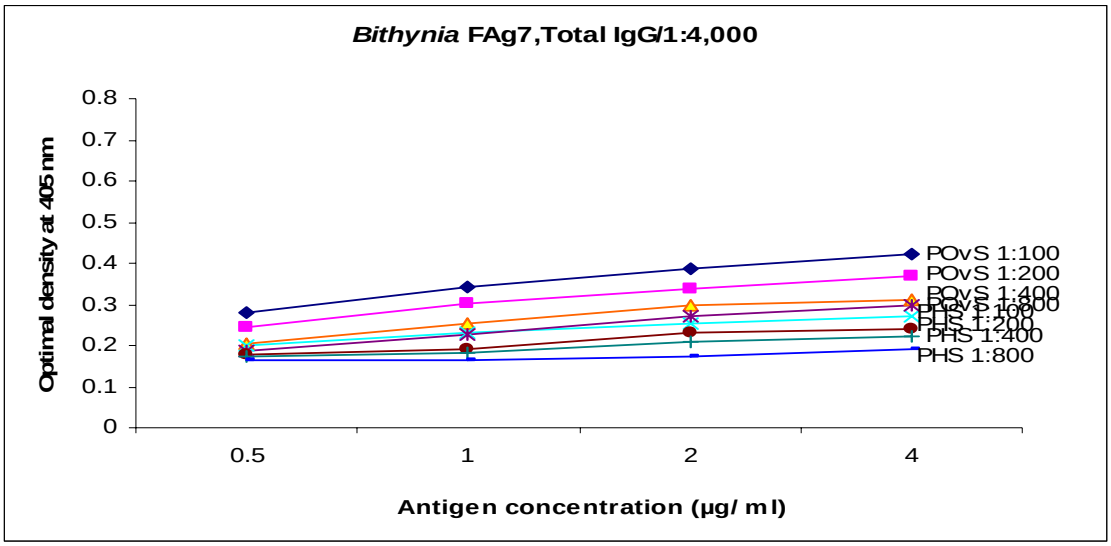

Figure 15: Checkerboard titration curves from the indirect ELISA for determination of the optimal concentration of Iso-FAg 7 and sera (POvS = pooled Opisthorchiasis serum and PHS = pooled health serum) by using peroxidase conjugate (total IgG) at 1:4,000

5.2 Determination of the antigens, reference serum and the conjugate (IgG subclass)

Using twenty iso-electricfocusing antigens of *Bithynia* snail fractions individual encountering with iso-type IgG1-4, it resulted in obtaining the selected iso-fractions following high OD-ratio values, namely, FAg2, FAg6, FAg7, and FAg10. Then, conditions of checkerboard titration were performed as followed: the *Bithynia* snail iso-antigens dilution to three concentrations of 1, 2, and 4 µg/ml, pool reference sera serially diluted from 1:100, 1:200, 1:400 and 1:800 and then using individual peroxidase conjugates (IgG1-4) at 1:500, 1:1,000 and 1:2,000.

Based on IgG1, the Iso- FAg 7 was selected to be analyzed for its proper conditions for full scale ELISA, presented in Figs. 16-18 The IgG2, IgG3, and IgG4 were reactive to Iso- FAg 6, Iso-FAg 2, and Iso-FAg 10, respectively. Their ELISA conditions were considered by checkerboard titrations in Figs. 19- 27.

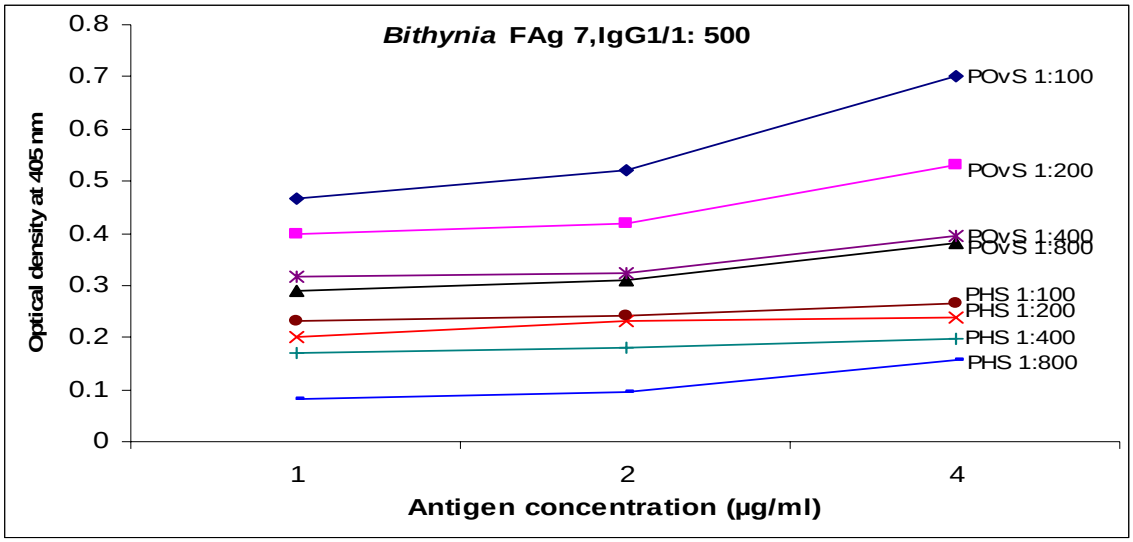

Figure 16: Checkerboard titration curves from the indirect ELISA for determination of the optimal concentration of Iso- FAg 7 and sera (POvS = pooled Opisthorchiasis serum and PHS = pooled health serum) by using peroxidase conjugate (IgG1) at 1:500

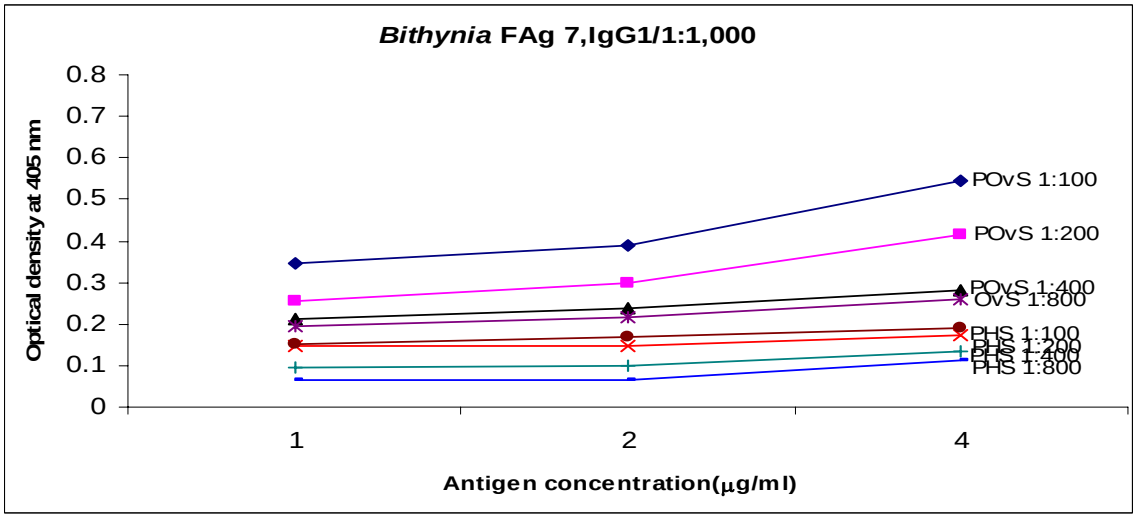

Figure 17: Checkerboard titration curves from the indirect ELISA for determination of the optimal concentration of Iso- FAg 7 and sera (POvS = pooled Opisthorchiasis serum and PHS = pooled health serum) by using peroxidase conjugate (IgG1) at 1:1,000

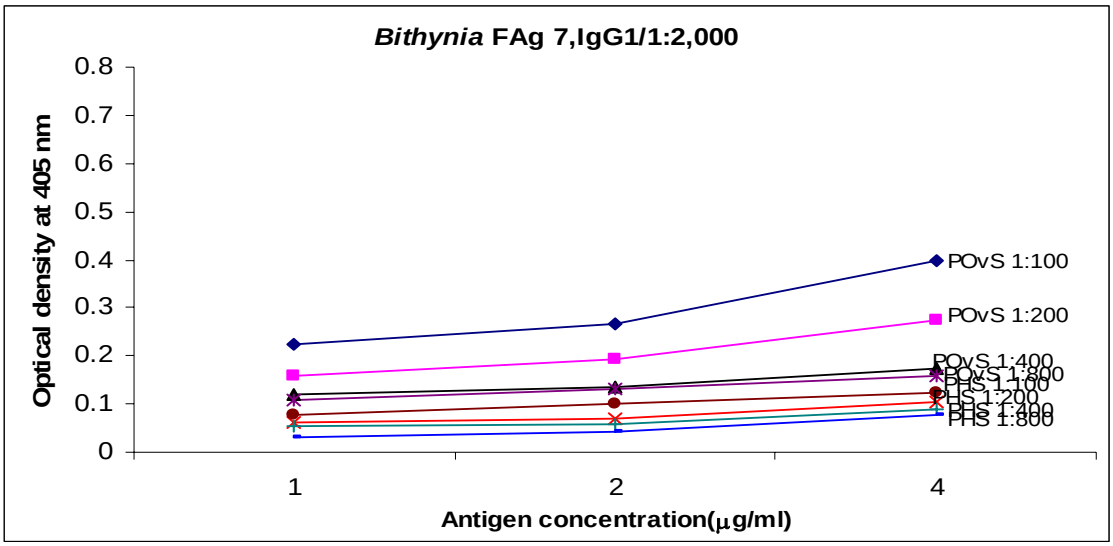

Figure 18: Checkerboard titration curves from the indirect ELISA for determination the optimal concentration of Iso- FAg 7 and sera (POvS = pooled Opisthorchiasis serum and PHS = pooled health serum) by using peroxidase conjugate (IgG1) at 1:2,000

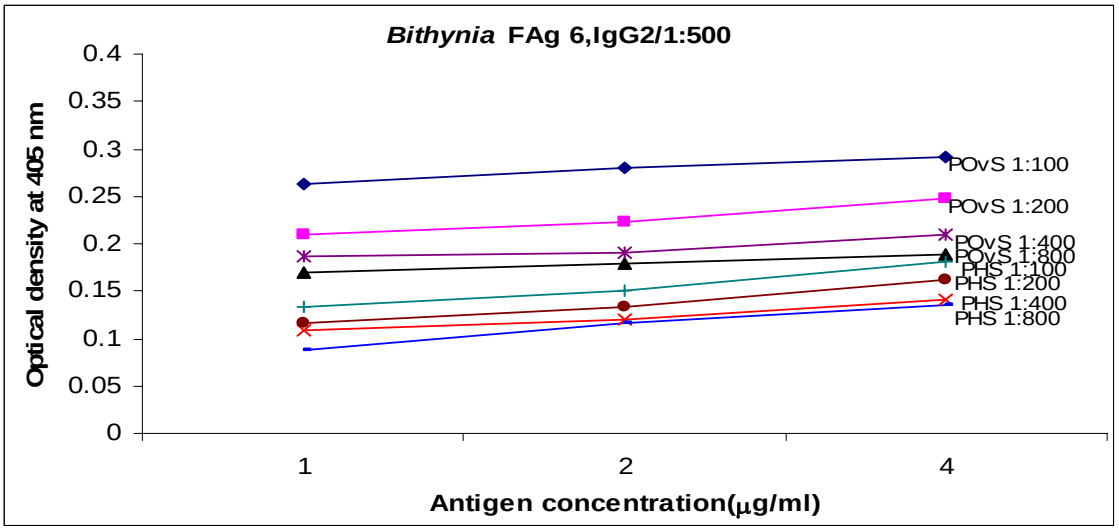

Figure 19: Checkerboard titration curves from the indirect ELISA for determination the optimal concentration of Iso-FAg 6 and sera (POvS = pooled Opisthorchiasis serum and PHS = pooled health serum) by using peroxidase conjugate (IgG2) at 1:500

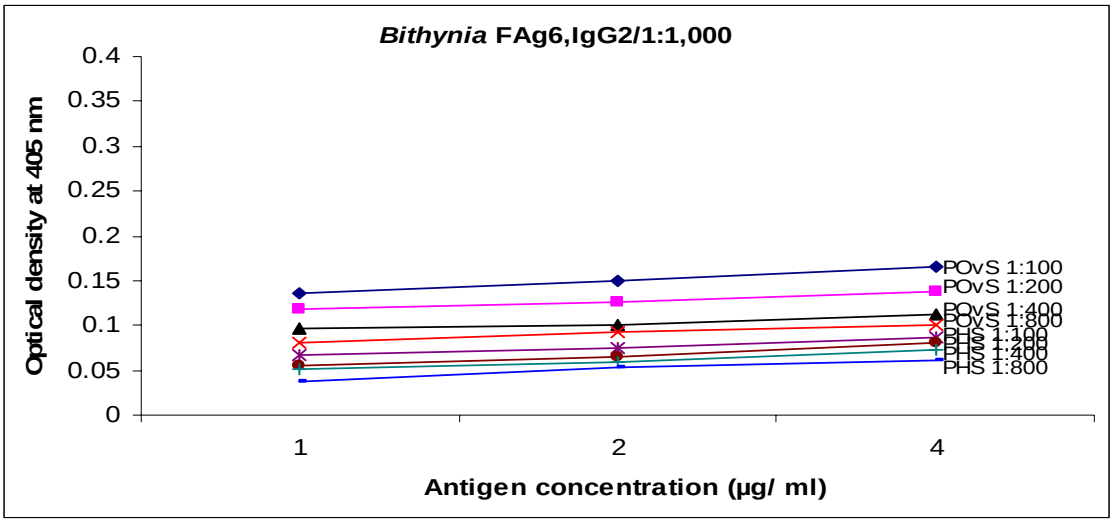

Figure 20: Checkerboard titration curves from the indirect ELISA for determination the optimal concentration of Iso- FAg 6 and sera (POvS = pooled Opisthorchiasis serum and PHS = pooled health serum) by using peroxidase conjugate (IgG2) at 1:1,000

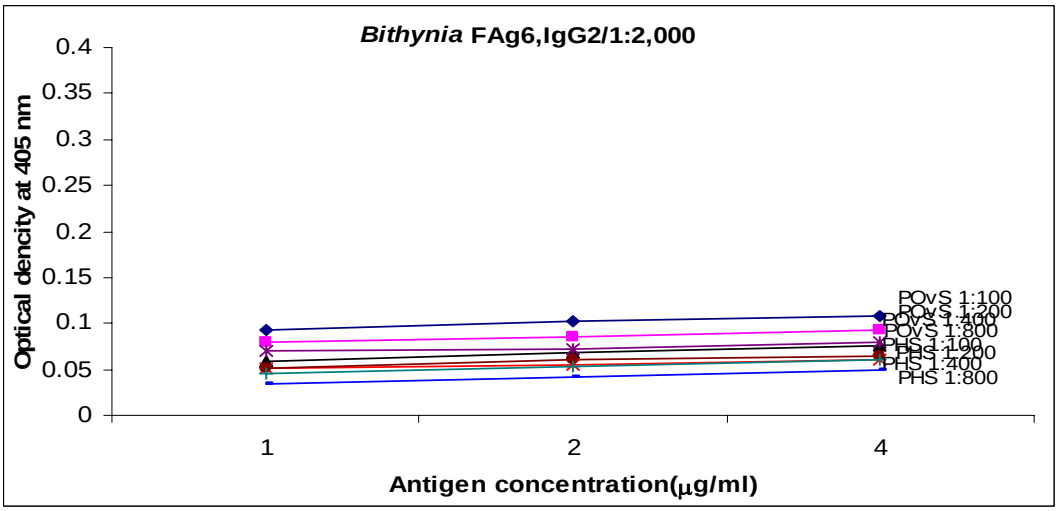

Figure 21: Checkerboard titration curves from the indirect ELISA for determination the optimal concentration of Iso- FAg 6 and sera (POvS = pooled Opisthorchiasis serum and PHS = pooled health serum) by using peroxidase conjugate (IgG2) at 1:2,000

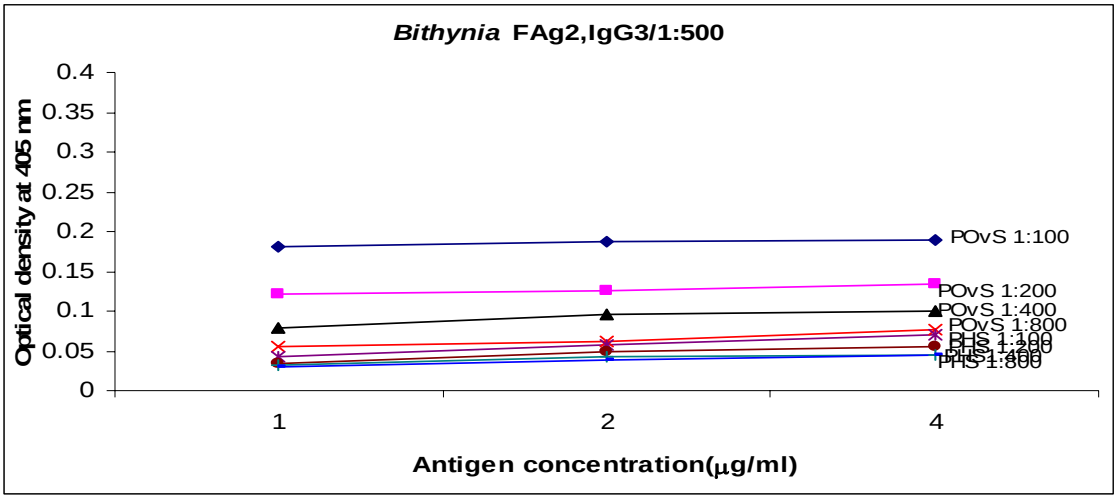

Figure 22: Checkerboard titration curves from the indirect ELISA for determination the optimal concentration of Iso- FAg 2 and sera (POvS = pooled Opisthorchiasis serum and PHS = pooled health serum) by using peroxidase conjugate (IgG3) at 1:500

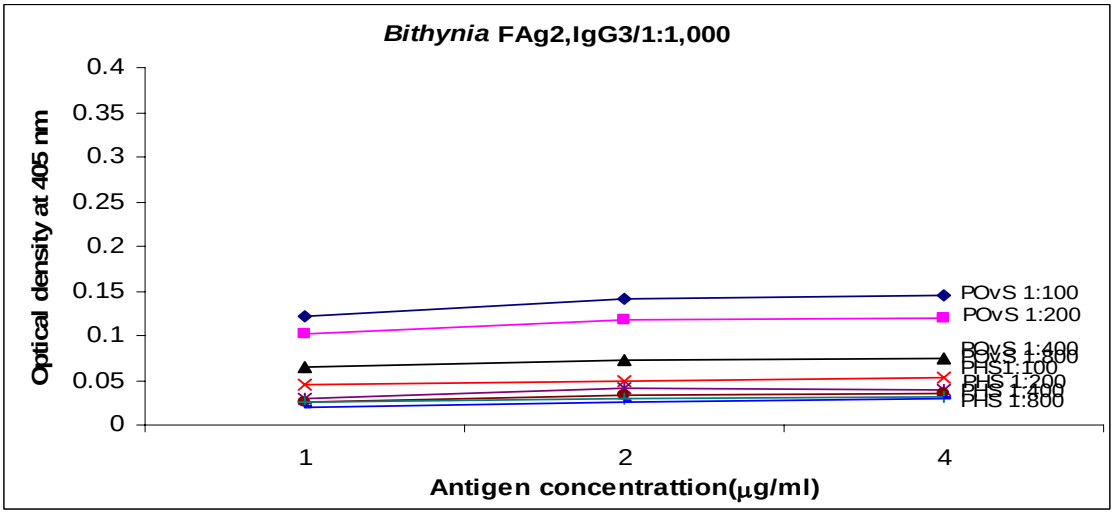

Figure 23: Checkerboard titration curves from the indirect ELISA for determination the optimal concentration of Iso- FAg 2 and sera (POvS = pooled Opisthorchiasis serum and PHS = pooled health serum) by using peroxidase conjugate (IgG3) at1:1,000

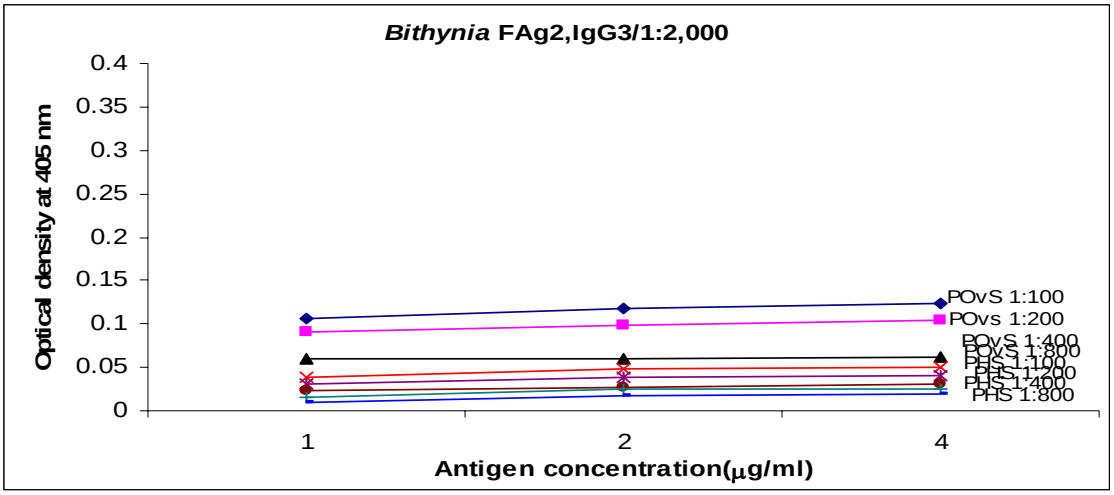

Figure 24: Checkerboard titration curves from the indirect ELISA for determination the optimal concentration of Iso- FAg 2 and sera (POvS = pooled Opisthorchiasis serum and PHS = pooled health serum) by using peroxidase conjugate (IgG3) at 1:2,000

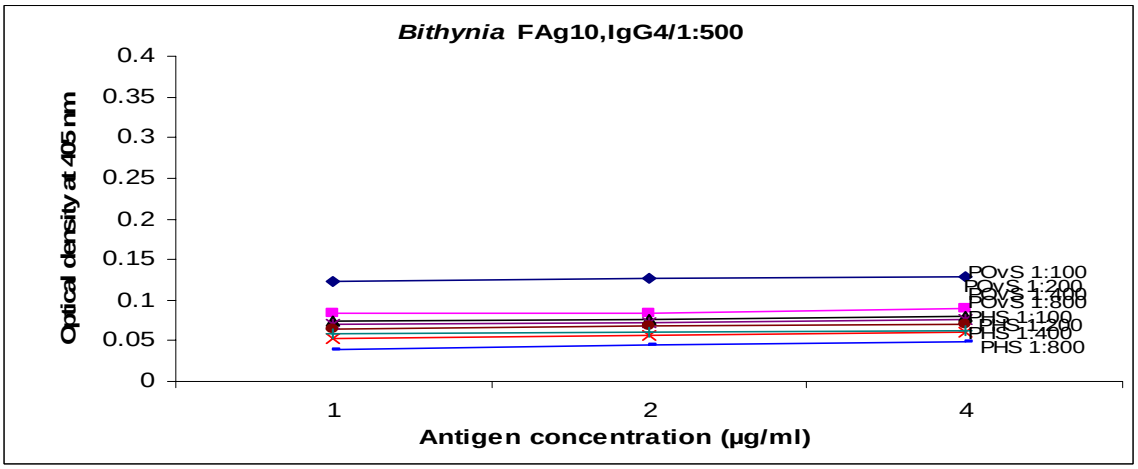

Figure 25: Checkerboard titration curves from the indirect ELISA for determination the optimal concentration of Iso- FAg10 and sera (POvS = pooled Opisthorchiasis serum and PHS = pooled health serum) by using peroxidase conjugate (IgG4) at 1:500

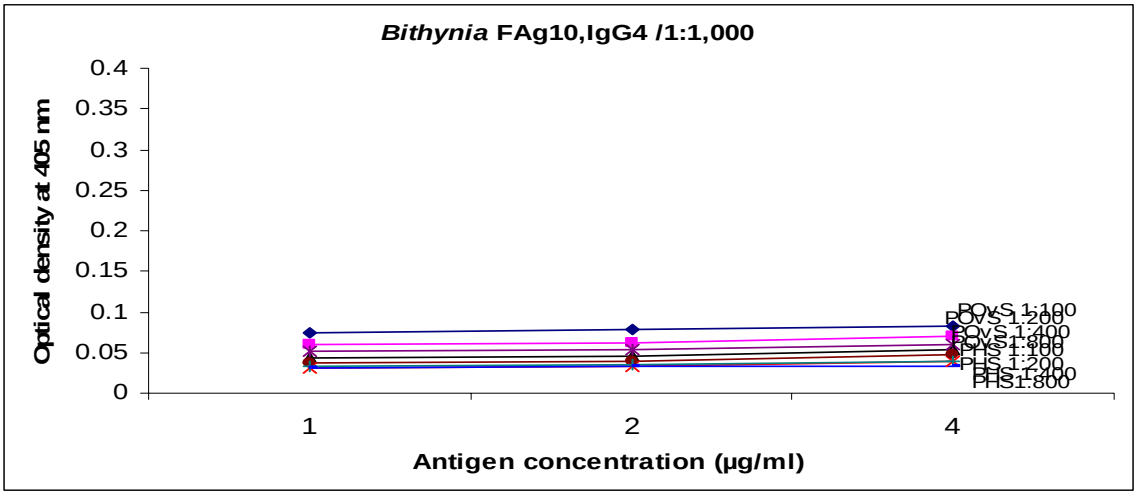

Figure 26: Checkerboard titration curves from the indirect ELISA for determination the optimal concentration of Iso-FAg10 and sera (POvS = pooled Opisthorchiasis serum and PHS = pooled health serum) by using peroxidase conjugate (IgG4) at 1:1,000

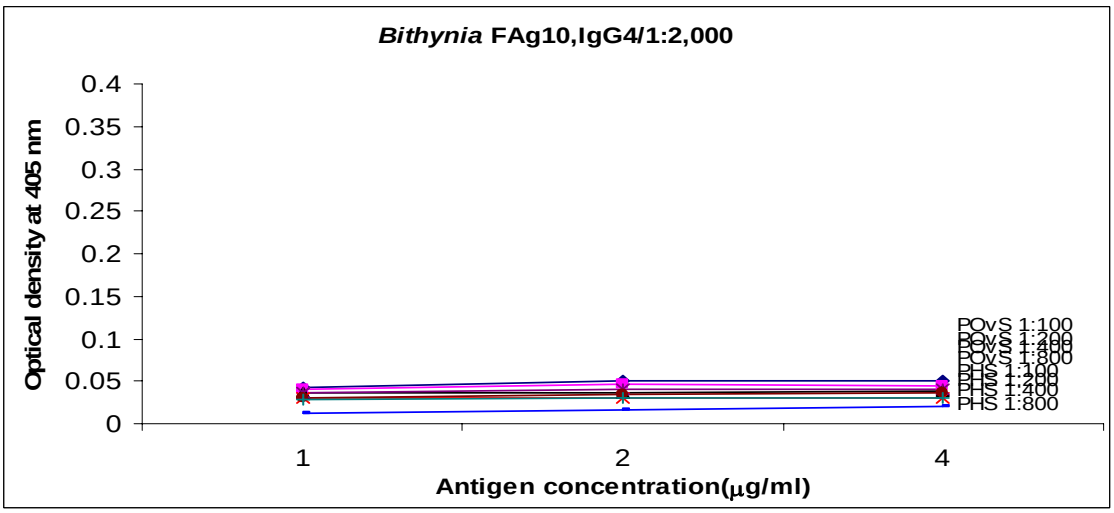

Figure 27: Checkerboard titration curves from the indirect ELISA for determination the optimal concentration of Iso-FAg 10 and sera (POvS = pooled Opisthorchiasis serum and PHS = pooled health serum) by using peroxidase conjugate (IgG4) at 1:2,000

It was found that all iso-fractionated antigens could react with antibodies from opisthorchiasis and healthy pooled sera, especially IgG2 to FAg 6, which their OD-values were extremely close to each other. IgG3 and IgG4 showed less OD-values separation between both pooled sera groups. IgG 1 to Iso-FAg 7 showed tendency on

OD-ELISA increasing, especially those of Opisthorchiasis pooled serum following higher microgram of antigen. However, four candidate antigens to IgG 1-4 were selected following their OD-values ratios.

After checkerboard titration, the candidate antigens have shown different concentration in each fractioned antigen. The appropriated concentrations are shown in table 4.

Table 4: Demonstration of optimal concentrations of serum, antigen, and peroxidase conjugation in each fractioned antigen

Selected Ag	IgG class	Serum dilution	Antigen (µg/ml)	Conjugate dilution
Iso-FAg7	Total	1:200	2	1:2000
Is0-FAg7	1	1:100	4	1:2000
Iso-FAg6	2	1:100	4	1:1000
Iso-FAg2	3	1:100	2	1:500
Iso-FAg10	4	1:100	2	1:500

6. Sensitivity, specificity and predictive values

In the evaluation of ELISA, sensitivity of all experiments was considered at the highest percentage lower than 100 percent following ELISA analysis due to the highest sensitivities of total IgG and IgG 3 were determined at X+1SD with 98% and 96%. Using *Bithynia* snail antigen Iso- FAg 7 to total IgG, mean OD-value and SD value of sera from 50 Opisthorchiasis patients were calculated by 0.655 and 0.113, respectively. The various cut off levels were determined from OD values of the healthy control group (Table 20). The mean OD-value and SD value of the healthy controls were 0.400 and 0.098, respectively (Table 20). A threshold value was considered at 0.498 (X+1SD) to calculate the sensitivity, specificity, positive and negative predictive values of the test. The results showed that 48 of 50 cases of Opisthorchiasis patients were 96% positive, and 50.00% (98/196 cases) of other parasitic infections were false positive and 13 of 35 cases of healthy control group (37.14%) showed false positive (Table 6). The sensitivity, specificity, positive and negative predictive values of test were 96%, 51.94%, 30.18%, and 98.36%,

respectively (Table 5, 21). The mean OD of Opisthorchiasis group was significant higher than the mean OD from sera of heterologous group ($p<0.05$) (Table 27). The OD values of other parasitic serum samples, which were higher than the cut off value, were shown in Table 7 and Fig 28.

Table 5: Demonstration of the cut off values, sensitivity, specificity, positive and negative predictive values of the ELISA using *Bithynia* snail Iso-FAg 7 encountering with total IgG.

cut-off value		Sensitivity (%)	Specificity (%)	Predictive value%	
SD	X+SD			Positive	Negative
1SD	**0.498**	**96**	**51.94**	**30.18**	**98.36**
2SD	0.596	84	73.16	40.38	95.48
3SD	0.694	24	88.74	42.85	84.36

Table 6: Number of ELISA positive sera of 3 groups tested against *Bithynia* snail antigen Iso- FAg 7 with total IgG at the cutoff value, X+1SD=0.498.

Group of test	No. of sera tested (cases)	No. of positive (%)
1.Opisthorchiasis	50	48(96.00%)
2.Healthy sera control	35	13(37.14%)
3.Other parasitic infection	196	98(50.00%)

Table 7: Demonstration of number and OD values of other parasitic infections over the cut off values at X+1SD=0.498.

No	Disease/control	Cases	False/ Poitive	OD values
1	Gnathostomiasis (Gn)	12	7	0.600, 0.615, 0.637, 0.654, 0.718,0.581,0.573
2	Strongyloidiasis (Ss)	10	4	0.626, 0.685, 0.758,0.570
3	Hookworm infection (Ho)	10	3	0.633,0.582,0.577
4	Trichinellosis (Tcn)	10	5	0.657,0.575,0.560,0.534,0.504
5	Capillariasis (Cp)	3	3	0.617,0.583,0.510
6	Toxocariasis (Tx)	10	1	0.614
7	Angiostrongyliasis (Ang)	10	6	0.639,0.593,0.574,0.555,0.549, 0.530
8	Ascariasis (As)	10	6	0.574,0.559,0.558,0.530,0.514,0.499
9	Trichuriasis (Tt)	10	8	0.580,0.501,0.500,0.4980.580, 0.501, 0.500,0.498
10	Bancroftian filariasis (Wb)	10	6	0.616,0.748,0.547,0.524,0.511,0.502
11	Enterobiasis (Ev)	3	1	0.584
12	Brugian filariasis (Bm)	10	2	0.543,0.536
13	Dirofilariasis (Df)	1	1	0.726
14	Neurocysticercosis (Ncc)	10	8	0.628,0.629,0.687,0.587,0.571, 0.554,0.543,0.502
15	Sparganosis (Sp)	3	2	0.630,0.555
16	Taeniasis (Ta)	10	5	0.637, 0.658,0.596,0.549,0.537
17	Echinococcosis (Ecc)	10	6	0.639, 0.6480.576,0.563,0.542, 0.533
18	Hymenolepiasis nana (Hm)	4	3	0.633,0.594,0.554
19	Paragonimiasis heterotremus (Pg)	10	5	0.634,0.657,0.665,0.744,0.577
20	Schistosomiasis (Sc)	4	3	0.611,0.598,0.526
21	Fasioliasis (Fas)	4	4	0.615, 0.634, 0.658,0.572
22	Haplochiasis (Ha)	10	9	0.605,0.608,0.616,0.658,0.664, 0.696,0.546,0.524,0.523
23	Creeping eruption (Cr)	3	-	-
24	Entamoebiasis (En)	3	-	-
25	Giardiasis (Gd)	4	-	-
26	Blastocystis homonis infection (Bh)	4	-	-
27	Malaria (Ma)	5	-	-
28	Toxoplasmiasis (Txp)	3	-	-

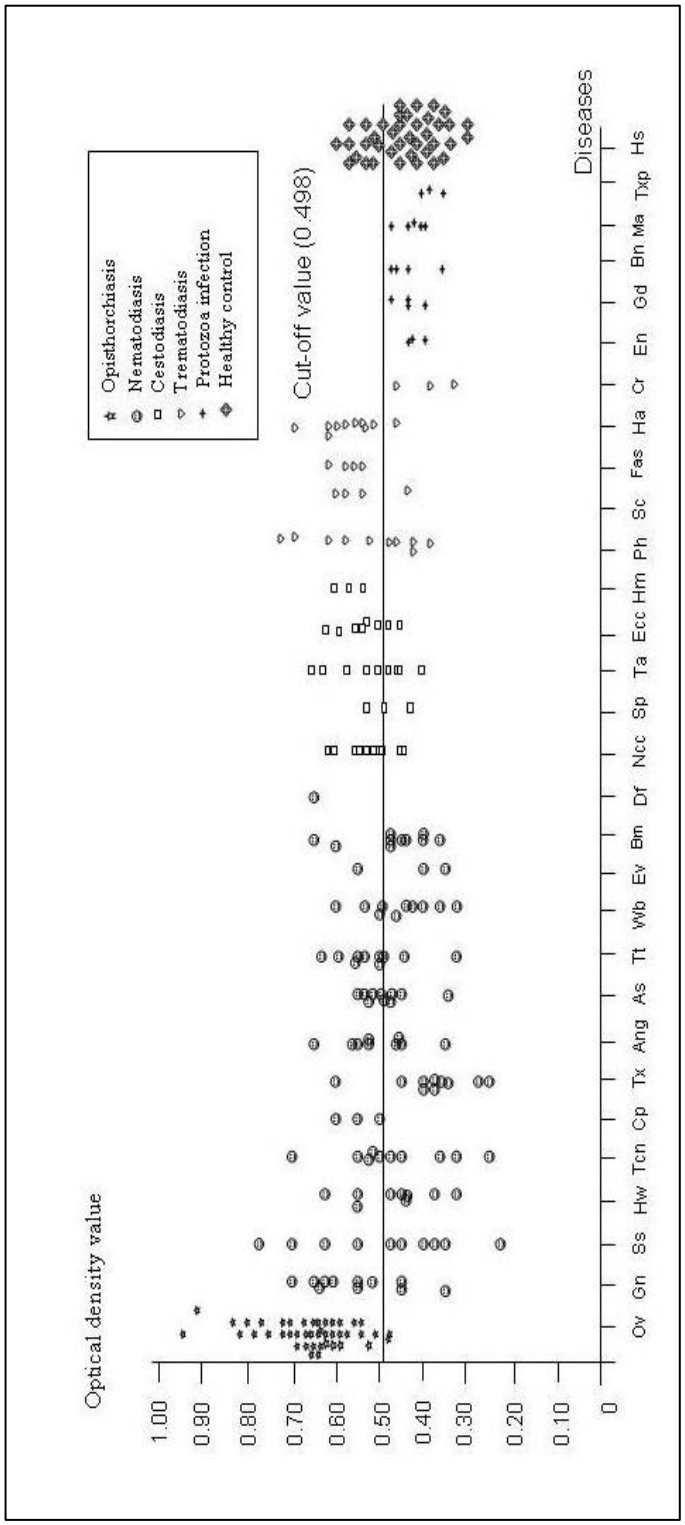

Figure 28: Scatter patterns of ELISA absorbance values of serum samples from various diseases and negative serum control. (*Bithynia* snail iso-FAg 7 with total IgG) Opisthorchiasis (Ov),Gnathostomiasis (Gn), Strongyloidiasis (Ss),Hookworm infection (Hw), Trichinellosis (Tcn), Capillariasis (Cp),Toxocariasis (Tx), Angiostrongyliasis (Ang), Ascariasis (As), Trichuriasis (Tt), Bancroftian filariasis (Wb), Enterobiasis (Ev), Brugian filariasis (Bm), Dirofilariasis (Df), Neurocysticercosis (Ncc), Sparganosis (Sp), Taeniasis (Ta), Echinococcosis (Ecc), Hymenolapiasis nana (Hm), Paragonimiasis heterotremus (Ph), Schistosomiasis (Sc), Fasioliasis (Fas), Haplochiasis (Ha), Creeping eruption (Cr), Entamoebiasis (En), Giardiasis(Gd), Blastocystis homonis infection (Bh), Malaria infection (Ma), Toxoplasmiasis (Txp), Healthy sera control (Hs)

Using iso-antigen Iso- FAg 7 with IgG1 detection, the Iso- FAg7 was determined for ELISA evaluation, i.e., mean OD value and SD value of sera from 50 Opisthorchiasis patients were calculated by 0.362 and 0.106, respectively. Then, various cut off levels were determined from OD values of the healthy control group (Table 20). The mean OD-value and SD value of the healthy controls were 0.065 and 0.038 respectively. A threshold value was considered at 0.221 (X+4SD) to calculate the sensitivity, specificity, positive and negative predictive values of the test (Table 8). The results showed that 49 of 50 cases of Opisthorchiasis patients were 98% positive, and 12.75% (25/196 cases) of other parasitic infections were false positive and all 35 cases of healthy control group showed true negative (Table 9). The sensitivity, specificity, positive and negative predictive values of test were 98%, 89.17%, 66.21%, and 99.51%, respectively (Table 8, 21). The mean OD of Opisthorchiasis group was significant higher than the mean OD from sera of heterologous group ($p<0.05$) (Table 23). It was observed that gnathostomiasis (2/12), Bancroftian filariasis (2/10), enterobiasis (1/3), neurocysticercosis (1/10), taeniasis (1/10), and haplorchiasis (1/10) show far higher OD values than the cut-off value meanwhile other false positives separated from those values. All OD values of other parasitic serum samples, which were higher than the cut off value, were shown in Table 10. True negative cases were found in strongyloidiasis, hookworm infection, capillariasis, toxocariasis, angiostrongyliasis, trichuriasis, Brugian filariasis, dirofilariasis, hymenolepiasis, creeping eruption, entamoebiasis, giardiasis, blastocystosis, malaria, and toxoplasmosis. However, OD values of these infections were high and loosely group (Fig. 29).

Table 8: Demonstration of the cut off values, sensitivity, specificity, positive and negative predictive values of the ELISA using *Bithynia* snail Iso-FAg7 encountering with IgG 1.

cut-off		Sensitivity (%)	Specificity (%)	Predictive value (%)	
SD	$\bar{X}$+SD			Positive	Negative
1SD	0.104	100	18.18	20.92	100
2SD	0.143	100	40.25	26.59	100
3SD	0.182	100	74.02	45.45	100
4SD	**0.221**	**98**	**89.17**	**66.21**	**99.51**
5SD	0.259	78	95.67	79.59	95.25

Table 9: Number of ELISA positive sera of 3 groups tested against *Bithynia* snail Iso-FAg 7 with IgG1 at the cut-off value, 0.221 ($\bar{X}$+4SD).

Group of test	No. of sera tested	No. of positive (%)
1. Opisthorchiasis	50	49 (98%)
2. Healthy sera control	35	0 (0%)
3. Other parasitic infection	196	25 (12.75%)

Table 10: Demonstration of number and OD values of other parasitic infections over the cut off value at X+4SD = 0.221.

No	Disease/control	Case	False Positive	OD values
1	Gnathostomiasis (Gn)	12	2	0.482, 0.490
2	Strongyloidiasis (Ss)	10	-	-
3	Hookworm infection (Hw)	10	-	-
4	Trichinellosis (Tcn)	10	1	0.249
5	Capillariasis (Cp)	3	-	-
6	Toxocariasis (Tx)	10	-	-
7	Angiostrongyliasis (Ang)	10	-	-
8	Ascariasis (As)	10	1	0.228
9	Trichuriasis (Tt)	10	-	-
10	Bancroftian filariasis (Wb)	10	2	0.339, 0.345
11	Enterobiasis (Ev)	3	1	0.467
12	Brugian filariasis (Bm)	10	-	-
13	Dirofilariasis (Df)	1	-	-
14	Neurocysticercosis (Ncc)	10	3	0.225, 0.417, 0.423
15	Sparganosis (Sp)	3	2	0.251, 0.269
16	Taeniasis (Ta)	10	2	0.250, 0.399
17	Echinococcosis (Ecc)	10	1	0.240
18	Hymenolepiasis nana (Hm)	4	-	-
19	Paragonimiasis heterotremus (Ph)	10	3	0.226, 0.230, 0.290
20	Schistosomiasis (Sc)	4	2	0.230, 0.252
21	Fasioliasis (Fas)	4	2	0.225, 0.240
22	Haplochiasis (Ha)	10	3	0.250, 0.291, 0.352
23	Creeping eruption (Cr)	3	-	-
24	Entamoebiasis (En)	3	-	-
25	Giardiasis (Gd)	4	-	-
26	Blastocystis homonis infection (Bh)	4	-	-
27	Malaria (Ma)	5	-	-
28	Toxoplasmiasis (Txp)	3	-	-

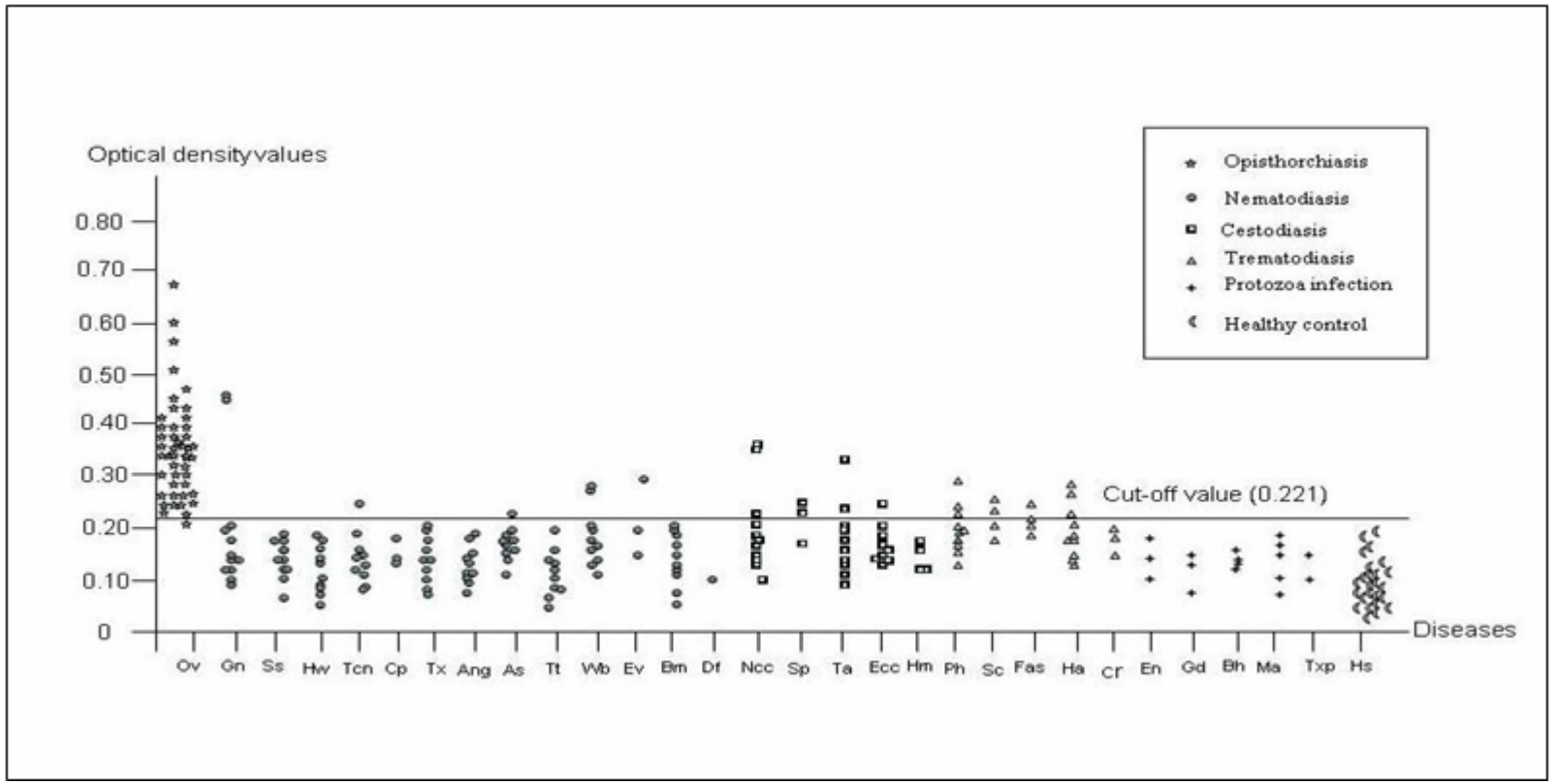

Figure 29: Scatter patterns of ELISA absorbance values of serum samples from various diseases and negative serum control. (*Bithynia* snail iso-FAg 7 with IgG 1) Opisthorchiasis (Ov),Gnathostomiasis (Gn), Strongyloidiasis (Ss),Hookworm infection (Hw), Trichinellosis (Tcn), Capillariasis (Cp), Toxocariasis (Tx), Angiostrongyliasis (Ang), Ascariasis (As), Trichuriasis (Tt), Bancroftian filariasis (Wb), Enterobiasis (Ev), Brugian filariasis (Bm), Dirofilariasis (Df), Neurocysticercosis (Ncc), Sparganosis (Sp), Taeniasis (Ta), Echinococcosis (Ecc), Hymenolapiasis nana (Hm), Paragonimiasis heterotremus (Ph), Schistosomiasis (Sc), Fasioliasis (Fas), Haplochiasis (Ha), Creeping eruption (Cr), Entamoebiasis (En), Giardiasis (Gd), Blastocystis homonis infection (Bh), Malaria infection (Ma), Toxoplasmiasis (Txp), Healthy sera control (Hs)

Using *Bithynia* snail antigen Iso- FAg 6 to IgG 2, mean OD value and SD value of sera from 50 Opisthorchiasis patients were calculated by 0.246 and 0.084 respectively. The various cut off levels were determined from OD values of the healthy control group (Table 20). The mean OD-value and SD value of the healthy controls were 0.096 and 0.024, respectively. A threshold value was considered at $\overline{0.192}$ (X+4SD) to calculate the sensitivity, specificity, positive and negative predictive values of the test (Table 11). The results showed that 45 of 50 cases of Opisthorchiasis patients were 90.00% positive, and 37.75% (74/196 cases) of other parasitic infections were false positive and 6 of 35 cases of healthy control group (17.14%) showed false positive (Table 12). The sensitivity, specificity, positive and negative predictive values of test were 90.00%, 77.05%, 45.91%, and 97.26%, respectively (Table 11, 21). The mean OD of Opisthorchiasis group was significant higher than the mean OD from sera of heterologous group ($p<0.05$) (Table 24). The OD values of other parasitic serum samples, which were higher than the cut off value, were shown in Table 13 and Fig 30.

Table 11: Demonstration of the cut off value, sensitivity, specificity, positive and negative predictive values of the ELISA using *Bithynia* snail Iso-FAg 6 encountering with IgG 2.

cut-off		Sensitivity %	Specificity %	Predictive value%	
SD	X+SD			Positive	Negative
1SD	0.120	100	14.28	20.16	100
2SD	0.144	100	27.70	23.04	100
3SD	0.168	100	51.51	30.86	100
4SD	**0.192**	**90**	**77.05**	**45.91**	**97.26**
5SD	0.216	78	94.80	76.47	95.21
6SD	0.240	60	98.26	88.23	91.63

Table 12: Number of ELISA positive sera of 3 groups tested against *Bithynia* snail Iso-FAg 6 with IgG 2 at the cutoff value, X+4SD=0.192

Group of test	No. of sera tested (cases)	No. of positive (%)
1.Opisthorchiasis	50	45(90.00%)
2. Healthy sera control	35	6(17.14%)
3.Other parasitic infection	196	74(37.75%)

Table 13: Demonstration of number and OD values of other parasitic infections over the cut off values at X+4SD=0.192.

No	Disease/control	Case	False Positive	OD values
1	Gnathostomiasis (Gn)	12	5	0.256, 0.372,0.207, 0.206, 0.199
2	Strongyloidiasis (Ss)	10	2	0.367, 0.200
3	Hookworm infection (Hw)	10	3	0.258, 0.301, 0.198
4	Trichinellosis (Tcn)	10	2	0.290, 0.201
5	Capillariasis (Cp)	3	1	0.192
6	Toxocariasis (Tx)	10	4	0.360, 0.390, 0.203, 0.198
7	Angiostrongyliasis (Ang)	10	3	0.370, 0.400, 0.199
8	Ascariasis (As)	10	3	0.250, 0.203, 0.197
9	Trichuriasis (Tt)	10	3	0.251, 0.234, 0.200
10	Bancroftian filariasis (Wb)	10	-	0.209, 0.200, 0.195
11	Enterobiasis (Ev)	3	-	0.259, 0.211
12	Brugian filariasis (Bm)	10	3	0.248,0.323, 0.220
13	Dirofilariasis (Df)	1	-	-
14	Neurocysticercosis (Ncc)	10	3	0.265, 0.283, 0.427
15	Sparganosis (Sp)	3	1	0.201
16	Taeniasis (Ta)	10	4	0.254, 0.531, 0.211, 0.200
17	Echinococcosis (Ecc)	10	6	0.222, 0.222, 0.271, 0.323, 0411, 0.210
18	Hymenolepiasis nana (Hm)	4	1	0.200
19	Paragonimiasis heterotremus (Ph)	10	6	0.290, 0.351, 0.342, 0.215, 0.200, 0.199
20	Schistosomiasis (Sc)	4	3	0.300, 0.412, 0.231
21	Fasioliasis (Fas)	4	3	0.211, 0.290, 0.409
22	Haplochiasis (Ha)	10	7	0.250, 0.250, 0.300, 0.331, 0.391, 0.202, 0.211
23	Creeping eruption (Cr)	3	2	0.261,
24	Entamoebiasis (En)	3	1	0.224
25	Giardiasis (Gd)	4	3	0.254, 0.233, 0.210
26	Blastocystis homonis infection(Bh)	4	1	0.220
27	Malaria (Ma)	5	2	0.221, 0.211
28	Toxoplasmiasis (Txp)	3	2	0.231, 0.266

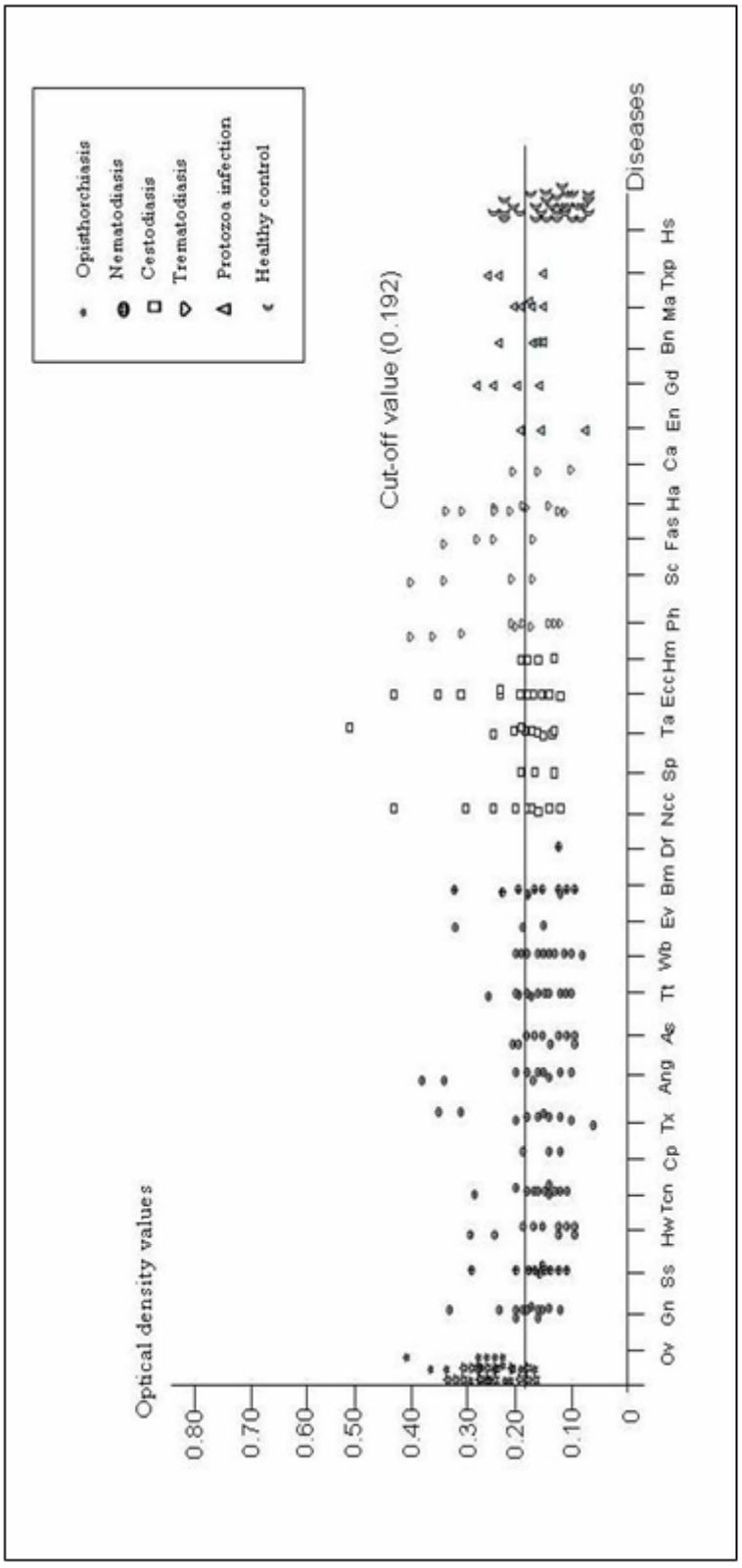

Figure 30: Scatter patterns of ELISA absorbance values of serum samples from various diseases and negative serum control. (*Bithynia* snail antigen FR 6 with IgG 2) Opisthorchiasis (Ov),Gnathostomiasis (Gn), Strongyloidiasis (Ss),Hookworm infection (Hw), Trichinellosis (Tcn), Capillariasis (Cp),Toxocariasis (Tx), Angiostrongyliasis (Ang), Ascariasis (As), Trichuriasis (Tt), Bancroftian filariasis (Wb), Enterobiasis (Ev), Brugian filariasis (Bm), Dirofilariasis (Df), Neurocysticercosis (Ncc), Sparganosis (Sp), Taeniasis (Ta), Echinococcosis (Ecc), Hymenolapiasis nana (Hm), Paragonimiasis heterotremus (Ph), Schistosomiasis (Sc), Fasioliasis (Fas), Haplochiasis (Ha), Creeping eruption (Cr), Entamoebiasis (En), Giardiasis (Gd),Blastocystis homonis infection (Bh), Malaria infection (Ma), Toxoplasmiasis (Txp), Healthy sera control (Hs)

Using *Bithynia* snail antigen Iso- FAg 2 to IgG3, mean OD value and SD value of sera from 50 Opisthorchiasis patients were calculated by 0.269 and 0.074 respectively. The various cut off levels were determined from OD values of the healthy control group (Table 20). The mean OD-value and SD value of the healthy controls were 0.154 and 0.033, respectively. A threshold value was considered at $\overline{0.187}$ (X+1SD) to calculate the sensitivity, specificity, positive and negative predictive values of the test (Table 14). The results showed that 48 of 50 cases of Opisthorchiasis patients were 96.00% positive, and 35.17% (70/196 cases) of other parasitic infections were false positive and 10 of 35 cases of healthy control group (28.57%) showed false positive (Table 15). The sensitivity, specificity, positive and negative predictive values of test were 96.00%, 65.36%, 37.50%, and 98.69%, respectively (Table 14, 21). The mean OD of Opisthorchiasis group was significant higher than the mean OD from sera of heterologous group ($p<0.05$) (Table 25). The OD values of other parasitic serum samples, which were higher than the cut off value, were shown in Table 16 and Fig. 31.

Table 14: Demonstration of the cut off value, sensitivity, specificity, positive and negative predictive values of the ELISA using *Bithynia* snail Iso-FAg 2 encountering with IgG 3.

cut-off		Sensitivity	Specificity	Predictive value%	
SD	X+SD	%	%	Positive	Negative
1SD	**0.187**	**96**	**65.36**	**37.50**	**98.69**
2SD	0.220	74	71.42	35.92	92.69
3SD	0.253	46	80.51	33.82	87.32
4SD	0.286	10	83.98	11.90	81.17

Table 15: Number of ELISA positive sera of 3 groups tested against *Bithynia* snail Iso- FAg 2 with IgG 3 at the cutoff value, X+1SD=0.187

Group of test	No. of sera tested (cases)	No. of positive (%)
1.Opisthorchiasis	50	48(96.00%)
2. Healthy sera control	35	10(28.57%)
3.Other parasitic infection	196	70(35.71%)

Table.16: Demonstration of number and OD values of other parasitic infections over the cut off values at X+1SD=0.187.

No	Disease/control	Cases	False Positive	OD values
1	Gnathostomiasis (Gn)	12	5	0.292, 0.388, 0.215, 0.200,0.197
2	Strongyloidiasis (Ss)	10	2	0.199,0.198
3	Hookworm infection (Hw)	10	1	0.198
4	Trichinellosis (Tcn)	10	3	0.286, 0.321,0.198
5	Capillariasis (Cp)	3	1	0.384
6	Toxocariasis (Tx)	10	1	0.216
7	Angiostrongyliasis (Ang)	10	4	0.283, 0.284, 0.351,0.193
8	Ascariasis (As)	10	3	0.274,0.201,0.194
9	Trichuriasis (Tt)	10	2	0.355,0.188
10	Bancroftian filariasis (Wb)	10	3	0.251,0.196,0.194
11	Enterobiasis (Ev)	3	2	0.237,0.200
12	Brugian filariasis (Bm)	10	3	0.235, 0.251,0.189
13	Dirofilariasis (Df)	1	-	-
14	Neurocysticercosis (Ncc)	10	4	0.238,0.216,0.199,0.194
15	Sparganosis (Sp)	3	-	-
16	Taeniasis (Ta)	10	7	0.284, 0.373, 0.411, 0.215, 0.215, 0.196, 0.194
17	Echinococcosis (Ecc)	10	1	0.238
18	Hymenolepiasis nana (Hm)	4	3	0.259, 0.321,0.194
19	Paragonimiasis heterotremus (Ph)	10	5	0.233, 0.341, 0.195, 0.190, 0.190
20	Schistosomiasis (Sc)	4	2	0.257, 0.311
21	Fasioliasis (Fas)	4	2	0.235,0.190
22	Haplochiasis (Ha)	10	8	0.230, 0.255, 0.257, 0.290, 0.311, 0.368,0.200,0.200
23	Creeping eruption (Cr)	3	3	0.242, 0.311,0.219
24	Entamoebiasis (En)	3	-	-
25	Giardiasis(Gd)	4	2	0.204,0.195
26	Blastocystic homonis infection(Bh)	4	2	0.255,0.201
27	Malaria (Ma)	5	-	-
28	Toxoplasmiasis (Txp)	3	1	0.243

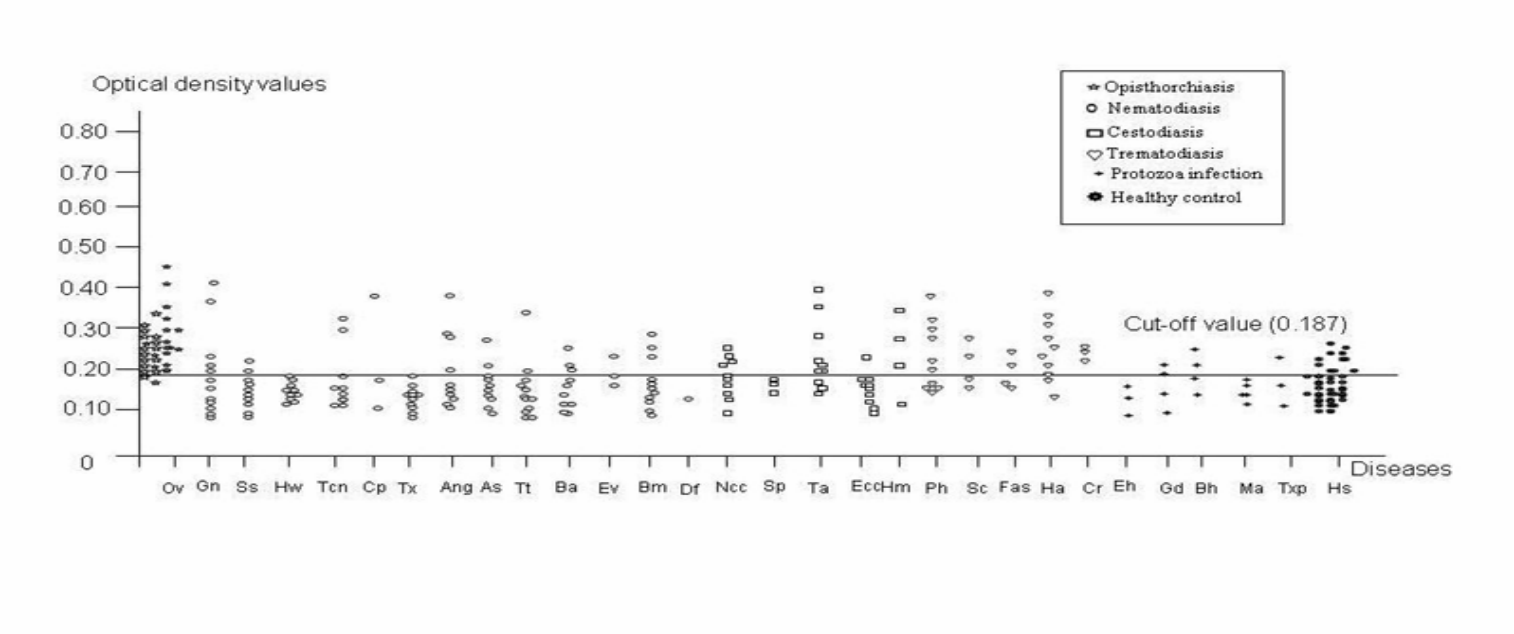

Figure 31: Scatter patterns of ELISA absorbance values of serum samples from various diseases and negative serum control (*Bithynia* snail Iso-FA2 with IgG3). Opisthorchiasis (Ov),Gnathostomiasis (Gn), Strongyloidiasis (Ss),Hookworm infection (Hw), Trichinellosis (Tcn), Capillariasis (Cp),Toxocariasis (Tx), Angiostrongyliasis (Ang), Ascariasis (As), Trichuriasis (Tt), Bancroftian filariasis (Wb), Enterobiasis (Ev), Brugian filariasis (Bm), Dirofilariasis (Df), Neurocysticercosis (Ncc), Sparganosis (Sp), Taeniasis (Ta), Echinococcosis (Ecc), Hymenolapiasis nana (Hm), Paragonimiasis heterotremus (Ph), Schistosomiasis (Sc), Fasioliasis (Fas), Haplochiasis (Ha), Creeping eruption (Cr), Entamoebiasis (En), Giardiasis (Gd),Blastocystis homonis infection (Bh), Malaria infection (Ma), Toxoplasmiasis (Txp), Healthy sera control (Hs)

Using *Bithynia* snail antigen Iso-FAg 10 to IgG 4, mean OD value and SD value of sera from 50 Opisthorchiasis patients were calculated by 0.298 and 0.108 respectively. The various cut off levels were determined from OD values of the healthy control group (Table 20). The mean OD-value and SD value of the healthy controls were 0.143 and 0.039, respectively. A threshold value was considered at 0.221 (X+2SD) to calculate the sensitivity, specificity, positive and negative predictive values of the test (Table 17). The results showed that 44 of 50 cases of Opisthorchiasis patients were 88.00% positive, and 16.32% (32/196 cases) of other parasitic infections were false positive and 2 of 35 cases of healthy control group (5.71%) showed false positive (Table 18). The sensitivity, specificity, positive and negative predictive values of test were 90.00%, 85.28%, 56.96%, and 97.52%, respectively (Table 17, 21). The mean OD of Opisthorchiasis group was significant higher than the mean OD from sera of heterologous group ($p<0.05$) (Table 26). The OD values of other parasitic serum samples, which were higher than the cut off value, were shown in Table 19 and Fig 32.

Table 17: Demonstration of the cut off value, sensitivity, specificity, positive and negative predictive values of the ELISA using *Bithynia* snail antigen Iso-FAg 10 encountering with IgG 4.

Cut-off		Sensitivity %	Specificity %	Predictive value%	
SD	X+SD			Positive	Negative
1SD	0.182	100	76.62	48.07	100
2SD	**0.221**	**90**	**85.28**	**56.96**	**97.52**
3SD	0.260	80	87.87	58.82	94.83
4SD	0.299	48	93.07	60.00	89.21

Table 18: Number of ELISA positive sera of 3 groups tested against *Bithynia* snail Iso- FAg 10 with IgG 4 at the cutoff value, X+2SD=0.221

Group of test	No. of sera tested (cases)	No. of positive (%)
1.Opisthorchiasis	50	44(88.00%)
2.Healthy sera control	35	2(5.71%)
3.Other parasitic infection	196	32(16.32%)

Table 19: Demonstration of OD values of other parasitic infections over the cut off values at X+2SD=0.221

No	Disease/control	Cases	False Positive	OD values
1	Gnathostomiasis (Gn)	12	2	0.233, 0.241
2	Strongyloidiasis (Ss)	10	1	0.237
3	Hookworm infection (Hw)	10	-	-
4	Trichinellosis (Tcn)	10	2	0.233, 0.300
5	Capillariasis (Cp)	3	1	0.231
6	Toxocariasis (Tx)	10	3	0.254, 0.327, 0.375
7	Angiostrongyliasis (Ang)	10	2	0.237, 0.264
8	Ascariasis (As)	10	1	0.288
9	Trichuriasis (Tt)	10	1	0.242
10	Bancroftian filariasis (Wb)	10	1	0.237
11	Enterobiasis (Ev)	3	-	-
12	Brugian filariasis (Bm)	10	2	0.258, 0.273
13	Dirofilariasis (Df)	1	-	-
14	Neurocysticercosis (Ncc)	10	2	0.239, 0.305
15	Sparganosis (Sp)	3		-
16	Taeniasis (Ta)	10	2	0.296, 0.338
17	Echinococcosis (Ecc)	10	-	-
18	Hymenolepiasis nana (Hm)	4	-	-
19	Paragonimiasis heterotremus (Ph)	10	3	0.236, 0.255, 0.303
20	Schistosomiasis (Sc)	4	2	0.237, 0.259
21	Fasioliasis (Fas)	4	1	0.248
22	Haplochiasis (Ha)	10	4	0.235, 0.256, 0.274, 0.286
23	Creeping eruption (Cr)	3	-	-
24	Entamoebiasis (En)	3	-	-
25	Giardiasis (Gd)	4	2	0.236, 0.284
26	Blastocystis homonis infection (Bh)	4	-	-
27	Malaria (Ma)	5	-	-
28	Toxoplasmiasis (Txp)	3	-	-

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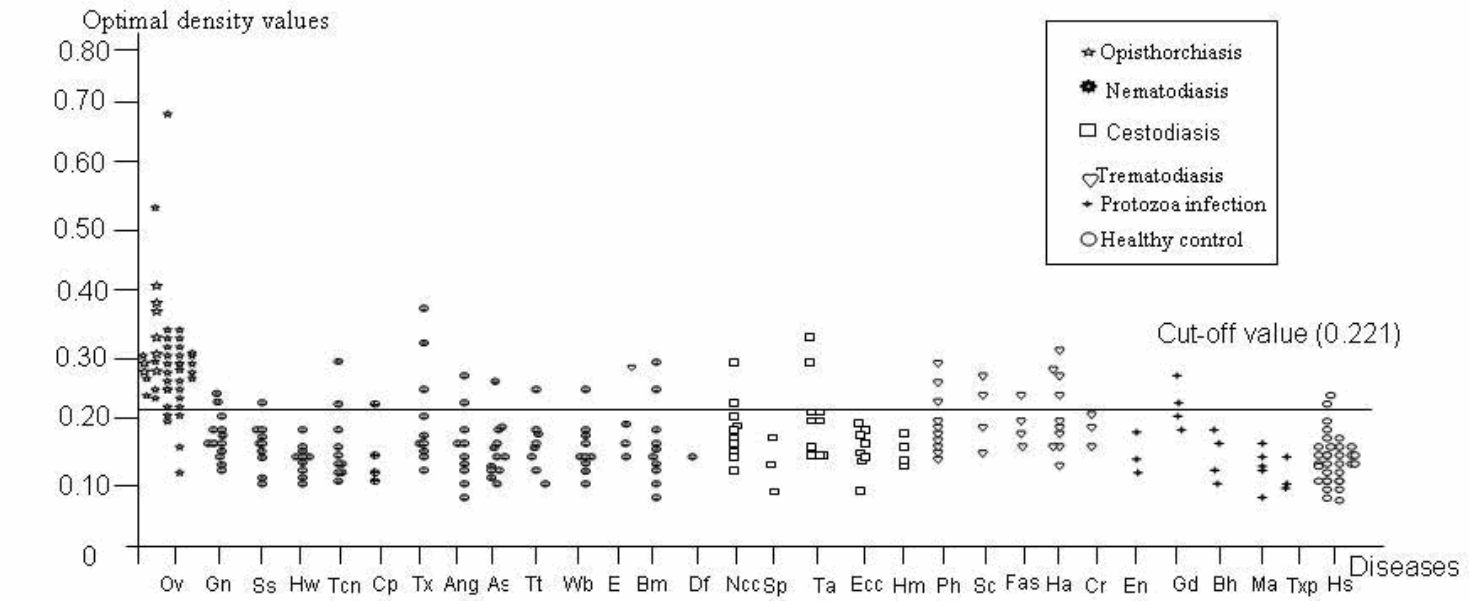

Figure 32: Scatter patterns of ELISA absorbance values of serum samples from various diseases and negative serum control. (*Bithynia* snail iso-FAg 10 with IgG 4) Opisthorchiasis (Ov),Gnathostomiasis (Gn), Strongyloidiasis (Ss),Hookworm infection (Hw), Trichinellosis (Tcn), Capillariasis (Cp),Toxocariasis (Tx), Angiostrongyliasis (Ang), Ascariasis (As), Trichuriasis (Tt), Bancroftian filariasis (Wb), Enterobiasis (Ev), Brugian filariasis (Bm), Dirofilariasis (Df), Neurocysticercosis (Ncc), Sparganosis (Sp), Taeniasis (Ta), Echinococcosis (Ecc), Hymenolapiasis nana (Hm), Paragonimiasis heterotremus (Ph), Schistosomiasis (Sc), Fasioliasis (Fas), Haplochiasis (Ha), Creeping eruption (Cr), Entamoebiasis (En), Giardiasis(Gd), Blastocystis homonis infection (Bh), Malaria infection (Ma), Toxoplasmiasis (Txp), Healthy sera control (Hs)

Table 20: The results showed differentiation of the mean ODs and Standard Deviation in among groups of Opisthorchiasis, heterologous, and healthy serum controls in each fractioned antigen

	FAg7/ IgG1	FAg6/ IgG2	FAg2/ IgG3	FAg10/ IgG4	FAg7/ Total IgG
Samples/Mean					
Opisthorchiasis	0.362	0.246	0.269	0.298	0.655
Heterologous group	0.217	0.113	0.204	0.150	0.419
healthy serum control	0.065	0.096	0.154	0.143	0.400
Samples/Std Deviation					
Opisthorchiasis	0.106	0.084	0.074	0.108	0.113
Heterologous group	0.068	0.045	0.047	0.047	0.107
healthy serum control	0.038	0.024	0.033	0.039	0.098

The finding of iso-fractioned Ag7 contained specific antigenic molecules to Opisthorchiasis antibody (IgG1), which gave a high mean of OD-ELISA of 0.362 and also very high sensitivity with 98%. Although mean and SD of heterologeous and healthy groups were high with 0.217 and 0.068, 0.065 and 0.038 but specificity was also high with 89.17%. It was surprisingly, IgG2 to all iso-fractioned antigens demonstrated non-OD separation between Opisthorchiasis pooled serum and healthy pooled serum as mentioned in the early results. Then, fractioned antigen 6 was selected by the highest OD-ratio with 0.018, which was lowest value when comparing with those selected antigen. These antigenic molecules were high with Opisthorchiasis antibody giving 90% sensitivity but also specific to antibodies from other parasitic infections and healthy control causing 65.80% respectively. Several higher OD-values than cut-off value, it was not reacted to low mean OD-values of heterologous and healthy control groups with 0.113 and 0.096, respectively.

Table 21: Accuracy, Sensitivity, Specificity and Positive- Negative predictive value of total IgG and IgG subclass antiboby to *Bithynia* snail iso-fractioned antigens for the serodiagnosis of human opisthorchiasis.

Fractioned Ag/ Types of IgG	Accuracy	Sensitivity	Specificity	Positive Predictive values	Negative Predictive values
iso-FAg7/1	90.75	98.00	89.17	56.75	100.00
iso-FAg6/2	80.43	90.00	65.80	36.29	96.77
iso-FAg2/3	78.29	74.00	80.63	36.84	99.31
iso-FAg10/4	85.77	88.00	83.98	54.32	97.46
isoFAg7/total IgG	81.14	84.00	79.22	46.66	95.76

Table 22: Summary of IgG subclasses and total IgG against *Bithynia* snail iso-fractioned antigen for the serodiagnosis of human opisthorchiasis and cross-reaction causing by serum samples from other parasitic infections and healthy control.

Group	Types of sera (No.)	FAg7/ Total IgG	Fag7/ IgG1	Fag6/ IgG2	FAg2/ IgG3	FAg10/ IgG4
A	Opisthorchiasis (50)	48	49	39	48	44
B	Gnathostomiasis (12)	7	2	5	5	2
	Strongyloidiasis (10)	4	0	2	2	1
	Hookworm infection (10)	3	0	3	1	0
	Trichinellosis (10)	5	1	2	3	2
	Capillariasis (3)	3	0	1	1	1
	Toxocariasis (10)	1	0	4	1	3
	Angiostrongyliasis (10)	6	0	3	4	2
	Ascariasis (10)	6	1	3	3	1
	Trichuriasis (10)	8	0	3	2	1
	Bancroftian filariasis (10)	6	2	3	3	1
	Enterobiasis (3)	1	1	2	2	0
	Brugian filariasis (10)	2	0	3	3	2
	Dirofilariasis (1)	1	0	0	0	0
	Neurocysticercosis (10)	8	3	3	4	2
	Sparganosis (3)	2	2	1	0	0
	Taeniasis (10)	5	2	4	7	2
	Echinococcosis (10)	6	1	6	1	0
	Hymenolepiasis nana (4)	3	0	1	3	0
	Paragonimiasis (10)	9	3	6	5	3
	Schistosomiasis (4)	3	2	3	2	2
	Fasioliasis (4)	4	2	3	2	1
	Haplochiasis (10)	9	3	7	8	4
	Creeping eruption (3)	0	0	1	3	0
	Entamoebiasis (3)	0	0	1	0	0
	Giardiasis(4)	0	0	3	2	2
	Blastocystis homonis (4)	0	0	1	2	0
	Malaria (5)	0	0	2	0	0
	Toxoplasmiasis (3)	0	0	2	1	0
C	Healthy control (35)	13	0	6	10	2
	Total (B+C)	115	25	111	80	34

Comparison among IgG 1-4 and their corresponding iso-fractioned antigens, IgG 1, eight from thirteen nematodiases did not bind with the FAg7 but IgG 2-4 from the some serum samples of nematodiases were more reactivity to FAgs 6, 3 and 10. All protozoan infectious and healthy sera did not react with FAg7 in IgG 1 detection,

and also total IgG, but 13 from 35 healthy control were cross-reaction total to IgG. Antibobies IgG 1-4 and total IgG from four trematodiases reacted with the corresponding iso-fractioned antigens from *Bithynia* snail intermediate host of *O. viverrini*.

7. Statistical analysis

One-way ANOVA was used for determining the OD-ELISA values in among groups of Opisthorchiasis, heterologous, and negative control to study the relationship and significant differentiation in each antigen.

Table 23: The results of statistical analysis were compared with among groups of Opisthorchiasis, heterologous, and negative control in Iso-FAG 7 and IgG1.

Iso-FAg 7/IgG1		mean difference	SD	F	Sig.	95% CI
Positive	Heterologous	0.23396*		232.246	0.000	0.2108-0.2571
	Negative	0.29665*	0.024		0.000	0.2645-0.3288

*The mean difference is significant at the .05 level.

Table 24: The results of statistical analysis were compared with among groups of Opisthorchiasis, heterologous, and negative control in Iso-FAG 6 and IgG2.

Iso-FAg 6/IgG2		mean difference	SD	F	Sig.	95% CI
Positive	Heterologous	0.13343*		137.585	0.000	0.1169-0.1500
	Negative	0.15093*	0.033		0.000	0.1280-0.1738

*The mean difference is significant at the .05 level.

Table 25: The results of statistical analysis were compared with among groups of Opisthorchiasis, heterologous, and negative control in Iso-FAg 2 and IgG3.

Iso-FAg 2/IgG3		mean difference	SD	F	Sig.	95% CI
Positive	Heterologous	0.06550*		53.433	0.000	0.0491-0.0819
	Negative	0.11509*	0.039		0.000	0.0924-0.1378

*The mean difference is significant at the .05 level.

Table 26: The results of statistical analysis were compared with among groups of Opisthorchiasis, heterologous, and negative control in Iso- FAg 10 and IgG4.

Iso-FAg10/IgG4		mean difference	SD	F	Sig.	95% CI
Positive	Heterologous	0.14690*		116.525	0.000	0.1275-0.1663
	Negative	0.15358*	0.098		0.000	0.1267-0.1805

*The mean difference is significant at the .05 level.

Table 27: The results of statistical analysis were compared with among groups of Opisthorchiasis, heterologous, and negative control in Iso- FAg 7 and total IgG.

Iso-FAg7/total IgG		Mean difference	SD	F	Sig.	95% CI
Positive	Heterologous	0.2356*		100.893	0.000	0.2019-0.2693
	Negative	0.2548*	0.038		0.000	0.2081-0.3015

*The mean difference is significant at the .05 level.

CHAPTER VI
DISCUSSION

Since the different *Opisthorchis viverrini* antigens have been used for diagnosis of opisthorchiasis viverrini, e.g., excretion-secretion (ES) product of adult worms (Amorpunt *et al*., 1991 Sirisinha *et al*., 1991, 1992, 1995; Sripa and Kaewkes, 2000), crude adult worm, egg, and metacercaria (Srivatanakul *et al*., 1985; Wongratanacheewin *et al*., 1988; Akai *et al*., 1995; Sakolvaree *et al*.,1997), oval antigen (Wongsarojt *et al*.,2001;Sripa and Kaewkes,2000), and snail antigen (Watthanapakulpanich *et al*.,1997; Waikagul *et al*., 2002). The cross-reaction with other parasitoses still occurrs and lead to misdiagnosis in routine work and also sensitivity of tests is included. Until now, a good test for opsithorchiasis has not yet been produced in the term of antibody detection.

As the concept of shared antigen between parasite and its intermediate host, Chanawong *et al*., (1990) initially used crude somatic antigens of three species of *Bithynia* snail, *Bithynai fumiculata, B. siamensis siamensis, B. siamensis goniomphalos* comparing with crude somatic antigen of *O. viverrini* adult worms showed snail antigens closely resemble results to each other. Thus, the *Bithynia* snails could be an antigen in antibody detection to opisthorchiasis. The shared antigens in the crude somatic extracts of three *Bithynia* species are compared with *O. viverrini* worm antigen by both SDS-PAGGE and Western blot analysis to select the best antigen. The results indicated the *B. s. goniomphalos* is the best antigen of snail antigen. It gave the result as good as *O. viverrini* product antigen with 80.2% and 81.2% sensitivity and specificity, respectively. However, it was still showed the cross-reactions with paragonimiasis and strongyliodiasis from eight different helminthiases (Wathanakulpanich *et al*., 1997). The shared antigen was done again to solve the cross-reaction and to increase sensitivity and specificity when the crude antigen from the *B. s. goniomphalos* was electroeluted at 53 kDa from polyacrylamide gel and

prepared a cocktail antigen by SephacrylS-200 HR gel filtration chromatography, and then analyzed by IgG-ELISA. Cross-reaction with eluted antigen was still occurred with strongyliodiasis and hymnolepiasis nana infection (Waikagul *et al*., 2002).

When study on the components of crude extracts of those *Bithynia* snails, there are several protein bands ranging their molecular weights from 13.5 to 90 kDa by using SDS-PAGE and stained with Coomassie brilliant blue. Approximately, 28 common protein bands are found among the extracts, i.e., 13.5, 16.5, 18, 19, 19.5, 20.5 22, 23.5, 25, 26.5, 27, 29, 30, 32, 37, 40, 42, 47, 50.5, 53, 55, 58.5, 61, 65.6, 74, 77, 84, and 90 kDa (Watthanakulpanich, Thesis, 1995). In our study, these isolated antigens are calculated as molecular weights, 89.50, 61.08, 53, 50.05, 40.62, 37.23, 32.27, 31, 29.25, 28.62, and 26.84 kDa, and few other bands, which seem to relate to those molecular weights of Watthanakulpanich's Thesis, (1995) but it might be different antigenic property. It is possible to subject crude *Bithynia* snail extract to be separated by gradient pH in liquid phase using iso-electric focusing technique, which movement of native protein molecules in the gradient condition will be stopped following their iso-electric point in a pH gradient.

This study aimed to obtain an improved *Bithynia* snail antigen by using advance technique, iso-electric focusing. The Iso-electric separation of crude antigen might limit cross-reaction from antibodies of other diseases meanwhile it is possible to believe that some reactive molecules to opisthorchiasis antibody may not be separated in gradient pH 3-10. Due to only 28.84% of iso-electric focusing antigen is recovered from 110 mg of crude antigen applied in the separation. In the other hand, Iso-FAg 1-20 vary in protein contents by analyzing with the same volume using SDS-PAGGE. Several protein bands were found in fraction 3-13 and very few in fractions 1, 2, and 14-20. The several protein bands and also strong detection with CBB were found in a range of iso-fractioned antigen 3-8 in pH 4.9-7.1. The finding is a little bit difference in a range of pH, based on research of Li *et al*., (2000) who reported difference of main protein bands of *Echinococcus multilocularis* occurring in the different pH, and pH range of 4 to 8.5 showed the high concentration of protein. When applying with the same microgram protein, CBB-stained banding pattern was very similar.

However, its antigenicity of each fraction could be predicted by OD-ELISA ratios. For IgG OD-ELISA ratios, Iso-FAgs 4-8 show higher separation of OD values between opisthorchiasis antibody and healthy control sera. In contrast, many iso-fractioned antigens have low antigenicity to opisthorchiasis antibody as shown in Iso-FAgs 1-3, and 9-20. It is observed that iso-FAg8, pH 7.2 contains high immunogenic molecules when determined by total IgG because high OD-ELISA value was 0.955 by antibody against *O. viverrini* worms but also high OD value, 0.274, with healthy control serum. This brings to a high OD ratio but it still lower than that of Iso-FAg7, pH 7.0. In this study, Iso-FAg7 gives the highest OD-ratio value, 3.6, although its OD value for pooled opisthorchiasis antibody (0.749) is lower than that (0.955) of Iso-FAg 8 but OD value (0.208) to pooled healthy control serum is lower than that (0.274) of Iso-FAg8. It means that iso-FAg7 has low antigenic property to antibody of pooled healthy controls. However, Iso-FAg7 is not good enough to opisthorchiasis detection because specificity of ELISA is 79.22% and 19.89% is false positive. In addition, a variety of separated biological samples, proteins and glycoproteins, each antigen that prepared by IFE gave difference of main protein bands at different pH depending on source of proteins. Protein from *Fasciola hepatica* indicated the highest concentration of protein in pH range of 4.6 to 9.3 (Chung *et al.*, 1993). Antigenic protein, which derived from a large particle fraction of muscle larvae of *Trichinella spiralis* showed 37 bands of protein on SDS-PAGGE. Most protein revealed within a pH range of 4.7-7.0, while all of the 22 glycoprotein had pH ranging from 4.0 to 6.5 (Despo and La, 1998). Moreover; Liana (2008) reported that *Biomphalaria tenagophila* protein was separated by IFE and most protein were identified in pH ranging from 3.85-7.18. These different pI ranges were defined and determined their immunoreactivity to serum, and might be useful to distinguish the reaction.

Our study on opisthorchiasis reacting with iso-electric focusing snail antigens, IgG subclasses related in different pHs, IgG1 to Iso-FAg7, at iso-electric point pH 7; IgG2 to Iso-FAg 6, pH 6.5; IgG3 to Iso-FAg 2, pH 4.1, and IgG4 to Iso-FAg 10, pH 7.3. It is observed that the selected iso-fractioned antigen (Iso-FAg2) at acidity, pH 4.1, to IgG3 shows the lowest sensitivity (74%). Although this antigen pH 4.1 is selected by the highest OD ratio but antigenic epitopes on those molecules are low

specific to antibody against *O. viverrini*. When observing OD-ELISA ratios of IgG1-4, all iso-fractioned antigens show unsatisfied discrimination between opisthorchiasis antibody and healthy control sera, especially IgG2 but a candidate of fractionated antigens to each IgG subclass must be selected for full scale ELISA. Based on selection for full scale ELISA, IgG1, 4, 3, and 2 response to four candidates of iso-FAgs 7, 10, 2, and 6 by high OD-ratios; 0.049, 0.047, 0.032, and 0.018, respectively. Although those antigen candidates can not give OD-ELISA ratios over 5 [good discrimination following ELISA theory should be in a range ratio, 5-10, by Voller *et al* (1979)] but the Iso-FAg7 can show high evaluation of test with 98% sensitivity and 89.17% specificity. It seems that separation by gradient pH can provide a number of antigenic molecules to opisthorchiasis and eliminate some of cross-reactive molecules. Besides, the checkerboards titration profile curves of IgG1, 2, 3, and 4 can help in selection of a good condition of each test, which would determine ratios of OD-ELISA values between pool positive and pool negative sera. Therefore, the same molecular weights of snail proteins in 20 fractions (shown in same microgram protein, Fig. 7) carry different antigenic property being indicated in OD-ELISA ratios, especially to total IgG, IgG1, IgG3, and IgG4 but not IgG2. Several publications using IEF techniques have tried to indicate the improved purified antigens for characterization and diagnosis of parasitic infections, e.g., onchocercosis (Klenk *et al*, 1983), cysticercosis (Guerra *et al*, 1982; Coker-Vann *et al*, 1984; Ko and Ng, 1998; Ito *et al*, 1998), and differential diagnosis of cystic and alveolar echinococcosis (Ito *et al*, 1999). A number of studies use IFE methods to separate and identify protein samples and probably solve the cross-reaction problem. In the purified *Taenia solium* cysticerci, the antigen B is appearently isoelectric at pH 8.6, but after purification under acidic conditions, it is isoelectric between pH 5 and 5.3. The use of antigen B in a serum ELISA results in the detection of 73% of NCC cases (Guerra *et al*, 1982). An IEF antigen, pH 6.3-6.5 immunologically reacted with 80% of cysticercosis cases but cross-reaction occurred from many cases of hydatidosis (Coker-Vann *et al*, 1984). Ko and Ng (1998) reported that fractioned antigens from cystic fluid of *Taenia solium* metacestodes using Rotofor cell were drastically reduced of cross-reactions in several pigs' heterologous sera. The good potential iso-fractioned antigens to serodiagnosis, F9-F15, showed more slightly cross-reaction with anti-*Ecchinococcus* sera in

immunoblot than cystic fluid antigen. Those antigenic fractions are negative double-antibody ELISA to other antisera against common parasites in their study. A good example of using Rotofor-separation, the charge antigens of cystic fluid, (fraction no.18, pH 9.6) and intact cyst (fraction no.19, pH 9.2) of *Taenia solium* metacestodes showed 100% sensitivity and specificity by ELISA (Ito *et al*, 1998). The IEF-fractionated antigens (10-26 kDa) from cystic fluid of *T. solium* metacestodes were prepared by isoelectric focusing technique. These antigens were found to be specific and sensitive for differential serodiagnosis of neurocysticercosis, especially from alveolar or cystic echinococcosis by ELISA and enzyme-linked immunoelectrotransfer blot (EITB) (Ito *et al*., 1998). In addition, the highly purified antigens were provided by single-step iso-electrofocusing using Rotofor cells and resulted in obtaining an antigen-B-rich fraction (8 kDa) from *Echinococcus granulosus* cyst fluid and an Em-18-rich fraction from *E. multilocularis* protoscolex extract. Both purified antigens are not cross-reacted with serum antibodies from 7 diseases, including hepatoma and normal controls in their study, especially cysticercosis, which other publications show cross-reaction with those antigens. Moreover, the good differential antigen is Em18 antigen, which can differentiate serum samples of alveolar echinococcosis from cystic echinococcosis. The antigen B is only genus-specific for *Echinococcus* (Ito *et al*, 1999).

The result of iso-FAg7 to IgG1 indicated 89.17% specificity, which cross-reaction occurred with all of trematode infections, paragonimiasis, schistosomiasis, fascioliasis, and haplorchiasis, cestode group, except hymenolepiasis, and few of nematode infections. Cross-reaction is not found in groups of protozoa infections and also in total IgG. The evidence of cross-reaction to Iso-FAg7 with total IgG is very serious in all helminthiases. It is possible that the antibodies between groups of opisthorchiasis and protozoa infections are less related to responsibility to antigenic epitopes of iso-FAg7. In addition, simple structures of protozoan may be involved in low antibody response to protozoan or it carries different antigenic epitopes from those of iso-FAg7. This is not seen in other fractions, iso-FAgs2, 6, 10, which are reactive to IgG3, IgG2, and IgG4. Although they are different iso-FAgs to IgG subclasses but it is indicated to IEF technique’s efficiency to separate some of cross-reactive molecules

as in the iso-FAg7. In the same explanation of pI separation, antibodies (IgG1) from nematode infections are also less reactive to iso-FAg7 but it is opposite in those fractions to IgG3, IgG2, and IgG4. The iso-FAg7 also has low specific epitopes to antibody IgG1 in healthy control sera and gives true negative but total IgG gives false positive to this antigen. Other three iso-FAgs2, 6, and 10 also create false positive to IgG3, IgG2, and IgG4, respectively.

CHAPTER VII
CONCLUSION

In the present study, iso-electricfocusing antigen of fraction7 (iso-FAg7) gives high sensitivity (98%) and specificity (89.17%) to IgG1 detection, which was based on selection of the highest OD-ELISA ratio in the comparison. Meanwhile, total IgG detection of the same fraction shows percent reactivity lower than those of IgG1 by sensitivity 84% and specificity 79.22%. The detection of IgG1 is better than other subclasses and total IgG but twenty five cases from thirteen helminthiases can react with this antigen. Therefore, a further study is possible to elute the selected reactive bands to opisthorchiasis sera, which differentiate from sera of normal control including those cases mentioned above. Secondly, all iso-fractioned antigens may be compared by selection of reactive fractioned antigens with opisthorchiasis sera differentiating from normal controls by antigen-antibody banding pattern of immunoblot. Only selected fractions should contain several antigenic epitopes to antibody against *O. viverrini* worms and be potential to provide good diagnosis of opisthorchiasis.

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APPPENDIX

APPENDIX A
ISOELECTRIC FOCUSING

Optimizing Fractionation

Reagent

Bio-Lyte® ampholytes prepared by Bio-Rad Laboratories, Inc.

1. Ampholyte choice

Bio-Lyte®ampholytes are supplied at concentrations of 40% (w/v), The final concentration of Bio-Lytes used in the Rotofor system depends on the protein concentration in a given sample:

Protein per milliliter	Bio-Lyte Ampholytes
>2mg	2%
1mg	1.5%
0.5mg	1.0%
0.25%	0.5%

2. Ampholyte concentration

Formula to determine the appropriate volume of a 40%Bio-lyte ampholyte solution to give a desired final concentration in a Rotofor sample

For the equation: $(C_1)(V_1)=(C_2)(V_2)$, solve for V_1

where C_1 = Staring concentration of Bio-Lyte (40%)

V_1 = Unknown volume of 40%Bio-Lyte to give desired final concentration.

C_2 = Final or desired concentration of Bio-Lyte

V_2 = Final volume of the sample to be applied to the Rotofor (35-58 ml) or Mini Rotofor (18 ml)

APPENDIX B
PROTEIN DETERMINATION

Reagents

1. Bovine serum albumin standards (2.0 mg/ml)

This chemical is commercially prepare by PIERCE, USA and stored at -10°C

2. Coomassie®plus protein assay reagent

This chemical reagent is commercially prepared by PIERCE, USA and stored at 4°C.

Protocol

1. Prepare a protein standard (standard working range = 20, 10, 5, 2.5, 1.25 and 0.625 μg/ml)

2. Prepare unknown samples in requirement dilution.

3. Add 150 μl of each protein standard, unknown samples and blank intoa microplate wells.

4. Add 150 μl of Coomassie®plus protein assay reagent to each well.

5. Read the absorbance value at 595 nm.

6. Construct a standard curve by plotting the average blank corrected 595 nm for each BSA standard versus its concentrations in μg/ml and using the standard curve to determine the protein concentration for each unknown sample.

APPENDIX C
SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GRADIENT GEL ELECTROPHORESIS (SDS-PAGE)

Reagent

1. Acrylamide: Bis-acrylamide solution

Acrylamide	30.0 g
Bis-acrylamide	0.8 g
DW to	100.0 g

This solution should be stored in a dark bottle at 4°C

2. 0.5 M Tris-HCl buffer, pH 6.8

Tris (Hydroxymethyl aminomethane)	6.05 g
DW	50.0 ml

The pH is adjusted to 6.8 with 1N HCl then DW is added up to 100 ml. The buffer is kept at 4°C.

3. 1.5 M Tris-HCl buffer, pH 8.8

Tris	18.15 g
DW	50.00 ml

The pH is adjusted to 8.8 with 1N HCl then DW is added up to 100 ml. The buffer is kept at 4°C.

4. 10% Sodium dodecyl sulfate (SDS)

SDS	10.0 g
DW	100.0 ml

The solution is kept at room temperature.

5. 10% Ammonium persulfate

Ammonium peroxodisulfate	1.0 g
DW	10.0 ml

This solution is freshly prepared as a stock solution and stored at 4°C.

6. N, N, N', N'-Tetra-methylethylenediamine (TEMED)

The solution is commercially prepared by Bio-Rad Laboratories and stored at 4°C.

7. Sample buffer (x3)

0.5M Tris-HCl buffer, pH 6.8	18.7 ml
SDS	4.5 g
Glycerol	30.0 ml
2-mercaptoethanol	15.0 ml
0.5%Bromophenol Blue	3.0 ml
DW to	100.0 ml

The buffer is stored in small plastic tubes at 4°C. Working sample buffer (1.5x) is prepared by diluting the 3x sample buffer with an equal volume of DW.

8. Electrode buffer or Tris-glycine buffer, pH 8.3

Tris	6.06 g
Glycine	28.80 g
SDS	2.0 g
DW to	2,000 ml

The buffer is kept at 4°C.

9. Gradient gel preparation

% Gel	Acrylamide :Bis (ml)	1.5M Tris-HCl, pH8.8 (ml)	10% SDS (ml)	10% Am per (ml)	TEMED (μl)	DW (ml)	Total (ml)
10%	1.20	0.88	27	13.5	2.0	1.40	3.5
12%	1.48	0.88	27	13.5	2.0	1.11	3.5
15%	1.75	0.88	27	13.5	2.0	0.83	3.5
17%	2.00	0.88	27	13.5	2.0	0.58	3.5
20%	4.00	1.50	46	23.0	3.5	0.40	6.0

10. 5% stacking gel

DW	1.50 ml
0.5M Tris-HCl, pH 6.8	0.63 ml
Acrylamide: Bis-acrylamide	0.40 ml

10% SDS	25.00 µl
10%Ammonium persulfate	12.50 µl
TEMED	2.50 µl

The solution is gently mixed and poured on the top of the gradient gels.

11. Coomassie brilliant blue stain

Coomassie brilliant blue R 250	5.0 g
Absolute methanol	400.0 ml
Glacial acetic acid	50.0 ml
Glycerol	25.0 ml
DW to	500.0 ml

The staining solution is kept at room temperature.

12. Destaining solution for gel

Absolute methanol	300 ml
Glacial methanol	50 ml
Glycerol	50 ml
DW to	625 ml

The solution is stored at room temperature.

APPENDIX D
ENZYME-LINKED IMMUNOSORBENT ASSAY
(INDIRECT ELISA)

1. Coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6)

0.05M Na_2CO_3

Na_2CO_3	0.53 g
DW to	100.0 ml

0.50 N NaHCO3

$NaHCO_3$	2.1 g
DW to	100.0 ml

To prepare this buffer, 2.1 g of NaHCO3 is dissolved in DW and the pH is adjusted to 9.6 with 0.05M Na_2CO_3 before the volume is addition of DW. The buffer is kept at 4 °C.

2. Phosphate buffer saline (PBS), pH 7.4

$NaH_2PO_4 \cdot$ H2O	4.14 g
$Na_2HPO4_2 \cdot$ H2O	30.0 g
NaCl	170.0 g
DW to	2000.0 ml

Working PBS solution (1X) is prepared by diluting 100 ml of 10X PBS solutions with 900 ml of DW.

3. Washing buffer (0.05% Tween 20 in phosphate buffer saline, PBS-T)

Tween 20	0.5 ml
PBS, pH 7.4 to	100.0ml

4. Blocking solution (0.5% BSA- 0.02% NaN_3)

BSA	0.5 g
NaN_3	0.1 ml
PBS, pH 7.4 to	100.0 ml

5. Substrate buffer (0.1 M citrate buffer, pH 4.5)

$Na_3C_6H_5O_7 \cdot 2H_2O$	2.94 g
Citric acid	2.10 g
DW to	100.0 ml

This buffer, is adjusted to pH 4.5 with 1NaOH, added to 100.0 ml by DW and stored at 4 °C

6. Substrate

2,2- azino-di-(3 ethyl- benzthiazoline sulfonate) (ABTS ,Sigma)

This chemical is dissolved in 1X PBS and mixed with 30% H_2O_2 before used.

7. Stop-reaction solution (1% SDS)

SDS	1 g
DW to	100 ml

This solution is stored at room temperature.

APPENDIX E

ANOVA
positive

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	7.78180	3	2.59393	256.1658	0.000
Within Groups	1.98470	196	0.01013		
Total	9.76650	199			

Table 28: ANOVA showed differentiation of sum of squares, *F*-value, mean square, and significant differentiation of between groups and within groups in fractioned and IgG subclasses (positive groups).

Multiple Comparisons
Dependent Variable: positive
LSD

		Mean Difference (I+J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
FR7+IgG1	FR6+IgG2	0.47818*	0.02013	0.000	0.4385	0.5178
	FR2+IgG3	0.45516*	0.02013	0.000	0.4155	0.4948
	FR10+IgG4	0.42764*	0.02013	0.000	0.3879	0.4673
FR6+IgG2	FR7+IgG1	-0.47818*	0.02013	0.000	-0.5178	-0.4384
	FR2+IgG3	-0.02302	0.02013	0.254	-0.0627	0.0166
	FR10+IgG4	-0.05054*	0.02013	0.013	-0.0902	-0.0108
FR2+IgG3	FR7+IgG1	-0.45516*	0.02013	0.000	-0.4948	-0.4154
	FR6+IgG2	0.02302	0.02013	0.254	-0.0166	0.0627
	FR10+IgG4	-0.02752	0.02013	0.173	-0.0672	0.0122
FR10+IgG4	FR7+IgG1	-0.42764*	0.02013	0.000	-0.4673	-0.3879
	FR6+IgG2	0.05054*	0.02013	0.013	0.0108	0.0902
	FR2+IgG3	0.02752	0.02013	0.173	-0.0122	0.0672

* The mean difference is significant at the .05 level.

Table 29: ANOVA showed differentiation of mean difference, significant differentiation, and 95% CI in among groups of antigen and among IgG subclasses (positive groups).

ANOVA
hetero

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.90354	3	0.30118	107.1101	0.000
Within Groups	2.11454	752	0.00281		
Total	3.01808	755			

Table 30: ANOVA showed differentiation of sum of squares, *F*-value, mean square, and significant differentiation of between groups and within groups in fractioned antigen and IgG subclasses (heterologous groups).

Multiple Comparisons
Dependent Variable: hetero
LSD

		Mean Difference (I+J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
FR7+IgG1	FR6+IgG2	0.01442*	0.00545	0.008	0.0037	0.0251
	FR2+IgG3	-0.07651*	0.00545	0.000	-0.0872	-0.0658
	FR10+IgG4	-0.02263*	0.00545	0.000	-0.0333	-0.0119
FR6+IgG2	FR7+IgG1	-0.01442*	0.00545	0.008	-0.0251	-0.0037
	FR2+IgG3	-0.09094*	0.00545	0.000	-0.1016	-0.0802
	FR10+IgG4	-0.03711*	0.00545	0.000	-0.0477	-0.0263
FR2+IgG3	FR7+IgG1	0.07651*	0.00545	0.000	0.0658	0.0872
	FR6+IgG2	0.09094*	0.00545	0.000	0.0802	0.1016
	FR10+IgG4	0.05388*	0.00545	0.000	0.0431	0.0645
FR10+IgG4	FR7+IgG1	0.02263*	0.00545	0.000	0.0119	0.0333
	FR6+IgG2	0.03715*	0.00545	0.000	0.0263	0.0477
	FR2+IgG3	-0.05388*	0.00545	0.000	-0.0645	-0.0431

* The mean difference is significant at the .05 level.

Table 31: ANOVA showed differentiation of mean difference, significant differentiation, and 95% CI in among groups of antigen and among IgG subclasses (heterologous groups).

ANOVA
negative

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.18411	3	0.06137	51.6295	0.000
Within Groups	0.16166	136	0.00119		
Total	0.34578	139			

Table 32: ANOVA showed differentiation of sum of squares, *F*-value, mean square, and significant differentiation of between groups and within groups in fractioned antigen and IgG subclasses (negative groups).

Multiple Comparisons
Dependent Variable: negative
LSD

		Mean Difference (I+J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
FR7+IgG1	FR6+IgG2	-0.03076*	0.00824	0.000	-0.0471	-0.0144
	FR2+IgG3	-0.08964*	0.00824	0.000	-0.1059	-0.0733
	FR10+IgG4	-0.07864*	0.00824	0.000	-0.0949	-0.0623
FR6+IgG2	FR7+IgG1	0.03076*	0.00824	0.000	0.0144	0.0471
	FR2+IgG3	-0.05885*	0.00824	0.000	-0.0751	-0.0425
	FR10+IgG4	-0.04788*	0.00824	0.000	-0.0641	-0.0315
FR2+IgG3	FR7+IgG1	0.08962*	0.00824	0.000	0.0733	0.1059
	FR6+IgG2	0.05885*	0.00824	0.000	0.0425	0.0751
	FR10+IgG4	0.01097*	0.00824	0.185	-0.0053	0.0272
FR10+IgG4	FR7+IgG1	0.07864*	0.00824	0.000	0.0623	0.0949
	FR6+IgG2	0.04788*	0.00824	0.000	0.0315	0.0641
	FR2+IgG3	-0.01097	0.00824	0.185	-0.0272	0.0053

* The mean difference is significant at the .05 level.

Table 33: ANOVA showed differentiation of mean difference, significant differentiation, and 95% CI in among groups of antigen and among IgG subclasses (negative groups).

ANOVA
IgG1

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.54484	2	1.27242	232.2459	0.000
Within Groups	1.48474	271	0.00548		
Total	4.02958	273			

Table 34: ANOVA showed differentiation of sum of squares, *F*-value, mean square, and significant differentiation of between groups and within groups in fractioned antigen number 7 and IgG 1.

Multiple Comparisons
Dependent Variable:
positive
LSD

		Mean Difference (I-J) (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
positive	heterologous	0.23396*	0.01177	0.000	0.2108	0.2571
	negative	0.29665*	0.01631	0.000	0.2645	0.3288
positive	heterologous	-0.23396*	0.01177	0.000	-0.2571	-0.2108
	negative	0.06269*	0.01362	0.000	0.0359	0.0895
positive	heterologous	-0.29665*	0.01631	0.000	-0.3288	-0.2645
	negative	-0.06269*	0.01362	0.000	-0.0895	-0.0359

* The mean difference is significant at the .05 level.

Table 35: ANOVA showed differentiation of mean difference, significant differentiation, and 95% CI among groups of positive, heterologous, and negative control in fractioned antigen number 7 and among IgG 1.

ANOVA
IgG2

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.76688	2	0.38344	137.5852	0.000
Within Groups	0.75526	271	0.00279		
Total	1.52214	273			

Table 36: ANOVA showed differentiation of sum of squares, *F*-value, mean square, and significant differentiation of between groups and within groups in fractioned antigen number 6 and IgG 2.

Multiple Comparisons
Dependent Variable: pos
LSD

		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
	(I-J)				Lower Bound	Upper Bound
positive	heterologous	0.13343*	0.00840	0.000	0.1169	0.1500
	negative	0.15093*	0.01163	0.000	0.1280	0.1738
positive	heterologous	-0.13343*	0.00840	0.000	-0.1500	-0.1169
	negative	0.01751	0.00971	0.073	-0.0016	0.0366
positive	heterologous	-0.15093*	0.01163	0.000	-0.1738	-0.1280
	negative	-0.01751	0.00971	0.073	-0.0366	0.0016

* The mean difference is significant at the .05 level.

Table 37: ANOVA showed differentiation of mean difference, significant differentiation, and 95% CI among groups of positive, heterologous, and negative control in fractioned antigen number 6 and among IgG2.

ANOVA
IgG3

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.29196	2	0.14598	53.43324	0.000
Within Groups	0.74037	271	0.00273		
Total	1.03233	273			

Table 38: ANOVA showed differentiation of sum of squares, *F*-value, mean square, and significant differentiation of between groups and within groups in fractioned antigen number 2 and IgG 3.

Multiple Comparisons
Dependent Variable: pos
LSD

		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
positive	heterologous	0.06550*	0.00831	0.000	0.0491	0.0819
	negative	0.11509*	0.01152	0.000	0.0924	0.1378
positive	heterologous	-0.06550*	0.00831	0.000	-0.0819	-0.0491
	negative	0.04959*	0.00962	0.000	0.0307	0.0685
positive	heterologous	-0.11509*	0.01152	0.000	-0.1378	-0.0924
	negative	-0.04959*	0.00962	0.000	-0.0685	-0.0307

* The mean difference is significant at the .05 level.

Table 39: ANOVA showed differentiation of mean difference, significant differentiation, and 95% CI among groups of positive, heterologous, and negative control in fractioned antigen number 2 and among IgG3.

ANOVA
IgG4

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.89607	2	0.44803	116.5253	0.000
Within Groups	1.04198	271	0.00384		
Total	1.93805	273			

Table 40: ANOVA showed differentiation of sum of squares, *F*-value, mean square, and significant differentiation of between groups and within groups in fractioned antigen number 10 and IgG 4.

Multiple Comparisons
Dependent Variable: IgGfour
LSD

		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
positive	heterologous	0.14690*	0.00986	0.000	0.1275	0.1663
	negative	0.15358*	0.01367	0.000	0.1267	0.1805
positive	heterologous	-0.1469	0.00986	0.000	-0.1663	-0.1275
	negative	0.00667*	0.01141	0.595	-0.0158	0.0291
positive	heterologous	-0.15358	0.01367	0.000	-0.1805	-0.1267
	negative	-0.00667*	0.01141	0.595	-0.0291	0.0158

* The mean difference is significant at the .05 level.

Table 41: ANOVA showed differentiation of mean difference, significant differentiation, and 95% CI among groups of positive, heterologous, and negative control in fractioned antigen number 10 and among IgG4.

ANOVA
FR7+total IgG

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.33911	2	1.16956	100.8933	0.000
Within Groups	3.14143	271	0.01159		
Total	5.48054	273			

Table 42: ANOVA showed differentiation of sum of squares, *F*-value, mean square, and significant differentiation of between groups and within groups in fractioned antigen number 7 and total IgG.

Multiple Comparisons
Dependent Variable:
positive
LSD

		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
positive	heterologous	0.2356*	0.01712	0.000	0.20195	0.26937
	negative	0.2548*	0.02373	0.000	0.20813	0.30156
positive	heterologous	-0.2356*	0.01712	0.000	-0.26937	-0.20195
	negative	0.0191	0.01981	0.333	-0.01983	0.05818
positive	heterologous	-0.2548*	0.02373	0.000	-0.30156	-0.20813
	negative	-0.0191	0.01981	0.333	-0.05818	0.01982

* The mean difference is significant at the .05 level.

Table 43: ANOVA showed differentiation of mean difference, significant differentiation, and 95% CI among groups of positive, heterologous, and negative control in fractioned antigen number 7 and among total IgG.

BIOGRAPHY

NAME : Mr. Wallop Pakdee
DATE OF BIRTH : 21 December 1972
PLACE OF BIRTH : Satun, Thailand
INSTUTION ATTEND : Ramkhamhaeng University
Bachelor degree of Science
(Biology)
POSITION HELD : Scientist
Department of Helminthology,
Faculty of Tropical Medicine,
Mahidol University
OFFICE ADDRESS : Department of Helminthology,
Faculty of Tropical Medicine,
Mahidol University
420/6 Ratchawithi Road,
Phayathai, Bangkok, 10400
Tel. +662-6435600