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ANTIBODY RESPONSES AND LYMPHOCYTE PROLIFERATION IN MICE EXPERIMENTALLY INFECTED WITH *Gnathostoma spinigerum*

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สุนิดา ไทยสม : การตอบสนองของแอนติบอดี และการเพิ่มจำนวนของลิมโฟซัยท์ในสัตว์ทดลองที่ติดเชื้อพยาธิตัวจี๊ด (ANTIBODY RESPONSES AND LYMPHOCYTE PROLIFERATION IN MICE EXPERIMENTALLY INFECTED WITH *Gnathostoma spinigerum*) อาจารย์ที่ปรึกษา: รศ. ไหม รัตนวรารักษ์, อาจารย์ที่ปรึกษาร่วม: รศ. วิไล ศักดิ์ศิริสัมพันธ์, 97 หน้า. ISBN 974-17-4423-4

พยาธิตัวจี๊ด เป็นหนอนพยาธิชนิดบุกรุกเนื้อเยื่อที่พบได้ในประเทศไทย ความเข้าใจเกี่ยวกับความสัมพันธ์ระหว่างโฮสต์กับปรสิตชนิดนี้ ในแง่ของการตอบสนองทางภูมิคุ้มกันยังไม่สมบูรณ์ การศึกษานี้เป็นการทดลองเกี่ยวกับการตอบสนองของแอนติบอดี และ การเพิ่มจำนวนของลิมโฟซัยท์ ในสัตว์ทดลองที่ติดเชื้อพยาธิตัวจี๊ด ทั้งก่อนและหลังการรักษาด้วยยาฆ่าพยาธิ ivermectin ที่ช่วงเวลาต่างๆ

นำตัวอ่อนเจริญเต็มขั้นระยะที่ 3 ซึ่งเป็นระยะติดต่อที่ได้จากปลาไหลในธรรมชาติ มาป้อนทางปากให้หนูทดลอง 2 กลุ่มๆ ละ 10 และ 20 larvae หลังจากนั้น 10 วัน นำหนูจำนวนครึ่งหนึ่งของแต่ละกลุ่มมาให้ยาฆ่าพยาธิด้วย ivermectin เพียงครั้งเดียวทางปาก ปริมาณ 0.4 มก./กก. ของน้ำหนักตัว แล้ววัดระดับแอนติบอดีด้วยวิธี ELISA ต่อ IgG, IgG1, IgG2a และ IgE จากน้ำเหลืองกลุ่มที่ติดเชื้อ พบว่าสามารถวัดได้ในวันที่ 10 หลังติดเชื้อ ระดับจะค่อยๆ เพิ่มขึ้น จนกระทั่งสูงสุดระหว่างวันที่ 60 และ 120 หลังติดเชื้อ จากนั้นจะเริ่มลดลงเรื่อยๆ จากการศึกษาพบว่า แอนติบอดีชนิด IgG1, IgG และ IgE ให้การตอบสนองมากเป็นสามอันดับแรกตามลำดับ ระดับของ IgG1 ในกลุ่มที่ให้ยาหลังจากติดเชื้อ 10 larvae จะมีระดับลดลงอย่างมีนัยสำคัญในวันที่ 90 ส่วนระดับของ IgE ในกลุ่มของหนูทดลองที่ติดเชื้อ 20 larvae จะมีระดับลดลงอย่างมีนัยสำคัญในวันที่ 90 หลังให้ยาเช่นกัน ในขณะที่ระดับ IgG ที่สูงขึ้นจากการติดเชื้อยังคงมีระดับสูงอยู่ตลอดการทดลองแม้จะได้รับยารักษาแล้ว 6 เดือน ส่วนระดับของ IgG2a ที่วัดได้พบว่ามีระดับต่ำมากในทุกกลุ่มของหนูทดลอง จากผลการทดลองน่าจะสามารถนำไปประยุกต์ใช้สำหรับการตรวจวินิจฉัยโรคทางห้องปฏิบัติการ และอาจนำไปใช้ประเมินประสิทธิภาพของการรักษาได้ ในส่วนของการเพิ่มจำนวนของลิมโฟซัยท์เมื่อนำเซลล์จากม้ามของหนูที่ติดเชื้อ 10 และ 20 larvae มาทดลองในวันที่ 10, 50 และ 180 หลังติดเชื้อ พบว่ามีการเปลี่ยนแปลงหรือแตกต่างจากกลุ่มควบคุมเมื่อกระตุ้นด้วย Con A โดยมีการลดลงแต่ไม่มาก และมีการไม่ตอบสนองต่อการกระตุ้นด้วยแอนติเจนจำเพาะ การตอบสนองต่อ Con A และ แอนติเจนนี้จะยิ่งผิดปกติหรือลดลงมากขึ้นเมื่อมีปริมาณการติดเชื้อที่รุนแรง และระยะเวลานาน การเปลี่ยนแปลงของการตอบสนองทางภูมิคุ้มกันนี้จะหายไป เมื่อพยาธิถูกกำจัดหรือพยาธิมีปริมาณลดลงด้วยยาฆ่าพยาธิ แสดงว่าความผิดปกติของการตอบสนองนี้สามารถกลับคืนมาได้ และมีความสัมพันธ์เกี่ยวข้องกับการติดเชื้อโดยตรง การศึกษานี้ช่วยสนับสนุนว่าการตอบสนองของ Th-2 ในการติดเชื้อพยาธิตัวจี๊ด มีส่วนสำคัญในการควบคุมการตอบสนองทั้งทางแอนติบอดี และการเพิ่มจำนวนของลิมโฟซัยท์ อย่างไรก็ตามเราจะต้องศึกษากลไกที่พยาธิตัวจี๊ดใช้ในการกด T cell proliferation ต่อไป

สาขาวิชา จุลชีววิทยาทางการแพทย์ (สหสาขาวิชา)
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ลายมือชื่อนิสิต สุนิดา ไทยสม
ลายมือชื่ออาจารย์ที่ปรึกษา ไหม รัตนวรารักษ์
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม วิไล ศักดิ์ศิริสัมพันธ์

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SUNIDA THAISOM: ANTIBODY RESPONSES AND LYMPHOCYTE PROLIFERATION IN MICE EXPERIMENTALLY INFECTED WITH *Gnathostoma spinigerum* THESIS ADVISOR : ASSOC. PROF. MAI RATTANAVARARAK, THESIS CO-ADVISORS : ASSOC.PROF. WILAI SAKSIRISAMPANT, 97 pp. ISBN : 974-17-4423-4

G. spinigerum is a tissue invasive nematode commonly found in Thailand. The host-parasite relationships of this parasitic infection especially the immune responses are not completely understood. To study the antibody responses and lymphocyte proliferation, the mouse model experimentally infected with this parasite were tested before and after treated with anthelmintic drug (ivermectin) at different time point.

The infective stage of advanced third stage larvae (L3s) obtained from natural infected eels was orally given to both groups of mice with 10 and 20 larvae. Ten days after infection, half of each group was treated with single oral dose of 0.4 mg/kg body weight ivermectin. The antibody levels (IgG, IgG1, IgG2a and IgE) from the sera could be detected within Day 10 post infection (PI) by ELISA. These antibodies level gradually increased, reached a plateau during Day 60 and 120 PI and then slightly decreased thereafter. From the overall kinetic responses, IgG1, IgG and IgE antibodies were the three top predominant isotypes. The level of IgG1 in the post drug treated (PRx) mice, infected with 10 larvae was statistic significantly decreased within Day 90 PRx. The IgE-Ab level of the 20 larvae infected mice was significantly lower within Day 90 after drug administration. While the IgG-Ab level still persisted and was not statistic significantly changed after treatment to the end of the experiment (6 month PRx). Poor response of IgG2a was noted in every time points of post infection and post drug treatment. These observations may lead to an application for immunodiagnosis and an evaluation of the effectiveness of chemotherapy. With regard to the lymphocyte proliferation, spleen cells from mice infected with 10 and 20 larvae were tested at Day 10, Day 50 and Day 180 PI. Altered reactivity (slightly depressed) to stimulation by Con A and unresponsiveness to specific stimulation by antigen were observed. The depressed responses were more obvious in animals with high dose and chronic infection. This phenomenon was abolished by anthelmintic treatment, suggesting that the altered and depressed responses were reversible and associated with active infection. The results from this study help confirm the role of Th-2 response in regulating antibody production and T cell proliferation response to *G.spinigerum*. Further studies are required to characterize the specific mechanism that *G.spinigerum* used to suppress T cell proliferation.

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Academic year 2005 Advisor's signature Mai Rattanavararak
Co-advisor's signature Wilai Saksirisampant

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Ultimately, I would like to convey my deepest gratitude to my parents for their continuous support, warmest understanding, and encouragement throughout this study.

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ABBREVIATIONS

Ab	antibody
Ag	antigen
BSA	bovine serum albumin
cm	centimeter
cpm	count per minute
Con A	concanavalin A
°C	degree celsius
DW	distilled water
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
et al.	et alli
g	gram
HCl	hydrochloric acid
^{3}H-Tdr	tritiated thymidine
IFN-γ	interferon gamma
Ig	immunoglobulin
IL	interleukin
kg	kilogram (s)
L	liter
L1s	first stage larvae
L2s	second stage larvae
L3s	third stage larvae
M	molar
mM	millimolar
mg	milligram (s)
mL	milliliter (s)
mm	millimeter (s)
min	minute (s)
NaCl	sodium chroride
NaOH	sodium hidroxide
No	number

PI	post infection
RPMI	Rosewell Park Memorial Institute
SD	standard deviation
SI	stimulation index
Th	T helper cell
2-ME	2-mercaptoethanal
μg	microgram
μl	microliter
%	percent

CHAPTER I

INTRODUCTION

Gnathostoma spinigerum is the major causative agent of human gnathostomiasis in Thailand. The parasite life cycle, which has cat and dog as the definitive hosts, involves two intermediate hosts. The first intermediate hosts are crustaceans and copepods. The second intermediate host are fresh water fish harboring the infective third-stage larvae (L3s)(Daengsvang, 1981).

Human are accidental hosts, acquiring the infection by consumption L3s in raw or undercooked fresh-water fish, especially swamp eel, eel, catfish and snake-headed fish (Daengsvang, 1981; Rojekittikhun et al., 1989; Saksirisampant et al., 2002).

The larva can migrate to any part of the human body and cause pathological changes in the tissues and organs, which it reaches. According to the similarity of the syndromes caused by other parasitic worms as well as the absence of eggs in human excreta, immunological techniques have until now become important as a diagnostic tool. In the past, many tests had been tried including detecting the skin reaction, and antibody with precipitation, radio-immunoassay, enzyme-linked immunosorbent assays and Western blot (Yamaguchi, 1952; Ando, 1957; Kasemsuth, 1981; Suntharasamai, 1985; Anantaphruti et al, 1986; Dharmkrong-at, 1986; Morakote, 1987; Maleewong, 1988; Tapchaisri et al., 1991). However, the results have demonstrated the false positive reaction with some other helminthic infections. Moreover, an evaluation of the effectiveness of chemotherapy as measured by ELISA changes, conversion of skin test, disappearance of swelling and return to normal of eosinophilia have shown limited potential use (Nontasut, 2000; Kraivichian et al., 2004). The host-parasite relationship in term of immune responses is still poorly understood. Much less information is currently available on both humoral and cellular immune status of this tissue nematode. On the other hand, the information of immune regulation of infections can indicate immune mechanism, and pathogenesis and can be applied to immunodiagnosis. When infection was established, both Th-1 and Th-2 response perform antagonistly to regulate the infection. In general the principle immune response to protozoa that survive within

macrophage is cell-mediated response, particularly macrophage activation by Th-1 cell-derived cytokines. Whereas in many helminthic infections, the responses are mediated by the activation of Th-2 subset of $CD4^+$ helper T cells. The activated Th-2 cells secrete IL-4 and IL-5 which IL-4 stimulates the production of the neutralizing antibodies (IgG1, IgG4 and IgE), while IL-5 stimulates the development and activation of eosinophils (Abbas and Lichtman, 2003). Limited evidences previously showed that human gnathostomiasis had an elevation of serum total IgE and specific IgE-Ab levels, and blood eosinophil (Soestayo et al., 1987; Saksirisampant et al., 2001; Kraivichian et al., 2004). A study on the immune responses of antibodies and lymphocyte proliferation of this mouse-gnathostomiasis is much of interesting. Antibodies level of IgG subclass and IgE-Ab can offer informations indicating the immuno-regulation and leading to an improvement of serodiagnosis and an evaluation of the effectiveness of chemotherapy. In addition, an in vitro detection of lymphocyte proliferation by stimulating with mitogen such as concanavalin A (Con A) and phytohemagglutinin (PHA) can confer the effector T cells function. Because in host-parasite interactions, parasites try to resist to immune effector mechanism or inhibit host immune response by multiple mechanisms. The defect of T cell function or T-cell anergy to parasite antigen and to the homologous antigens has been observed in many infections included *Trichinella spiralis* (Faubert, 1982), *Onchocerca volvulus* (Soboslay et al., 1999), *Toxocara canis* (Allen et al., 1996) and *Taenia crassiceps* (Sciutto et al., 1995). This generalized immunodepression is possibly related to Th-2 responses, which lead to down-regulation of host-immune responses, by the induction of IL-4 (Allen et al., 1996).

In this research, two groups of mice were infected orally with 10 and 20 larvae of L3s. Ten days after infection, half of them were treated with ivermectin to evaluate the effect of the anthelmintic drug. An ELISA and lymphoproliferative stimulation by Con A had been used to compare the antibody responses and the cellular response between infected and uninfected; with or without ivermectin administration. The ELISA antibody responses of IgG, IgG1, IgG2a and IgE and the immunodepression of infected and drug treated mice could be shown in this study.

CHAPTER II

OBJECTIVES

1. To study the antibody responses in experimental infected mice of both before and after anthelmintic treatment by

    1.1 Studying the kinetics of the IgG-Ab, IgG-Ab subclass 1 and subclass 2a and IgE-Ab levels (ELISA) to L3s somatic Ag in mice infected with 10 and 20 larvae of *G.spinigerum* compare to normal control group at different time points (0, 10, 30, 60, 90, 120 and 180 days).

    1.2 Detecting the IgG-Ab, IgG-Ab subclass 1 and subclass 2a and IgE-Ab levels (ELISA) to L3s somatic Ag in post ivermectin treated mice infected with 10 and 20 larvae of *G.spinigerum* compare to normal control and the corresponding pretreatment groups at different time points (0, 10, 30, 60, 90, 120 and 180 days).

2. To study the lymphocyte proliferation in experimental infected mice of both before and after anthelmintic treatment by

    2.1 Studying the specific and non specific lymphocyte proliferative responses of spleen cells from mice infected with 10 and 20 larvae of *G.spinigerum* compare to the normal control groups at difference time points (0, 10, 50 and 180 days).

    2.2 Detecting the specific and non specific lymphocyte proliferative responses of spleen cells from post ivermectin treated mice infected with 10 and 20 larvae of *G.spinigerum* compare to normal control and the corresponding pretreatment groups at difference time points (0, 10, 50 and 180 days).

CHAPTER III

LITERATURE REVIEWS

1. Biology of *Gnathostoma spinigerum*

1.1 Geographical distribution

Infection caused by *Gnathostoma spinigerum* is common in many Asian countries including Thailand, China, Japan, Vietnam, Myanmar, India, Bangladesh, Malaysia, Indonesia, Israel and Philippines (Rojekittikhun et al., 1989; Rusnak and Lucey, 1993; Edward et al., 1999) with a few reported cases in Australia (Moorhouse et al., 1970) Africa (Chhuon et al., 1976), Mexico and Ecuador (Ollague et al., 1984; Ogata et al., 1998).

1.2 Classification

Gnathostomiasis is a zoonotic disease caused by roundworms of the genus Gnathostoma. There are 23 species have been reported (Almeyda, 1991). Only five species of mature adult Gnathostoma were natually recovered from the stomachs and urinary system of animals in Southeast Asia. These are *G.spinigerum, G.hispidum, G.doloresi, G.vietnamicum* and *G.malaysiae.*

Phylum Nematoda
Subclass Secernentea
Order Spirurida
Suborder Spirurina
Superfamily Gnathostomatoidea (Thelaziodea)
Family Gnathostomatidea
Subfamily Gnathostomatinea
Genus Gnathostoma

1.3 Life cycle

G.spinigerum is the only causative agent for human infection in Thailand (Daengsvang, 1980; Radomyos, 1987; Kamiya, 1987; Rojekittikhun, 2002). Adult *G. spinigerum* was first discovered by Richard Owen in 1836 from gastric tumours of a young tiger that died in the London Zoological Garden (Daengsvang, 1980).

The first case of human gnathostomiasis was reported when the worm was recovered from a swelling in the breast of a young Thai women living in Bangkok (Daengsvang, 1983). Thereafter increasing number of cases with removed *G.spinigerum* were reported from many countries (Morishita, 1924; Rojekittikhun et al. 1989; Akahane et al., 1994).

Prommas and Daengsvang firstly described the life cycle of *G.spinigerum* in 1933. Cats, dogs, tigers, lions and leopards can act as the definitive host. Fresh water cyclops (*Mesocyclops leickarti, Eucyclops agilis, Cyclops varicans* and *Thermocyclops sp.*) are the first intermediate hosts. A wide variety of animals including fishes, birds, reptiles, amphibians and some mammals for example the domestic poultry, frog, snake are the second intermediate hosts. Adult male and female worms inhabit a stomach of the definitive host which they produce non-embryonated eggs. The eggs are released through the opening of the stomach tumor and pass out the lumen in the faeces. First-stage larvae (L1s) develop and hatch from the eggs within 12 days in fresh water at 25-31 ^{o}C. The actively swimming first-stage larvae are then ingested by species of first intermediate host, in which they penetrate the gut to the hemocoel to become complete second-stage larvae (L2s). Further developments occur inside the body cavity, and the larvae finally transform into early third stage (L3s) within 6-7 days (Daengsvang, 1986).

When the infected cyclops are eaten by fish or other second intermediate hosts, the larvae develop into advanced L3s in the flesh of these animals. Forty-eight species of vertebrates have been reported to serve naturally as the second intermediate or paratenic hosts. These are fish (20 species), amphibians (2 species), reptiles (11 species), avian (11 species) and mammals (4 species). Among these animals, swamp eels (*Monopterus albus,* previously *Fluta alba*) was the best second intermediate of *G.spinigerum* on the basis of having the highest prevalence rate and the greatest infection intensity (Daengsvang, 1980; Rojekittikhun et al., 2002; Saksirisampant et al., 2002). The maximum number of gnathostome larvae collected as ever been recorded was 2,582 larvae from one eel in Nakhon Nayok Province (Setasuban et al., 1991). Almost all of the gnathostome larvae recovered from eels have been in their encysted form. About half (43%-52%) of the total number of larvae were concentrated in the liver, whereas the remaining half were in the whole muscles (Rojekittikhun, 2002). The definitive hosts get infection by eating these infected fleshes. The taken up larvae will penetrate the stomach wall, enter the liver,

and migrate to muscle and connective tissues where they molt to immature worms. The worms return to the stomach by penetrating the stomach wall from the serosal side and become mature adults in the cavity of a tumor-like mass which communicates with the stomach lumen through a small opening. It took about 6-7 months for the worm to become sexually mature.

Man is an accidental host, because after the L3s being ingested, the parasite cannot fully develop. The larvae then could move to various following organs: namely respiratory tract, gastrointestinal tract, genito-urinary tract, mucous membrane of the soft palate, central nervous system and eyeball (Kittiponghansa, 1987; Prijyamonda, 1955; Bodhidat, 1956).

From a total of 23 worms obtained from patients attending various hospitals and were sent to the Faculty of Tropical Medicine for identification (Radomyos, 1987), 5 were advanced L3s (21.7%), 14 were immature worms (60.9%) and 4 were mature worms (17.4%). The size of these advanced L3s varied from 2.20-3.50 mm x 0.40-0.63 mm. The immature worms were those of 8 males and 6 females with the average size of 4.63-9.35 mm x 0.60-1.05 mm and 3.8-16.2 mm x 0.83-1.0 mm respectively. The mature worms were all male whose size varied from 9.9-12.5 mm x 1.0-1.25 mm (Daengsvang, 1980).

There are thirty species of vertebrates that are experimentally susceptible to be accidental hosts or second intermediate hosts. Those are 2 species of fresh-water fish, 5 species of amphibian, 5 species of reptile, 3 species of avian, 2 species of crustacean and 13 species of mammal (Norway rat, white rat, black rat, little house rat, white mouse, hamster, guinea pig, tree shrew, squirrel, domestic pig, Macaque monkeys and leaf monkey)(Daengsvang, 1980; Rojekittikhun, 2002).

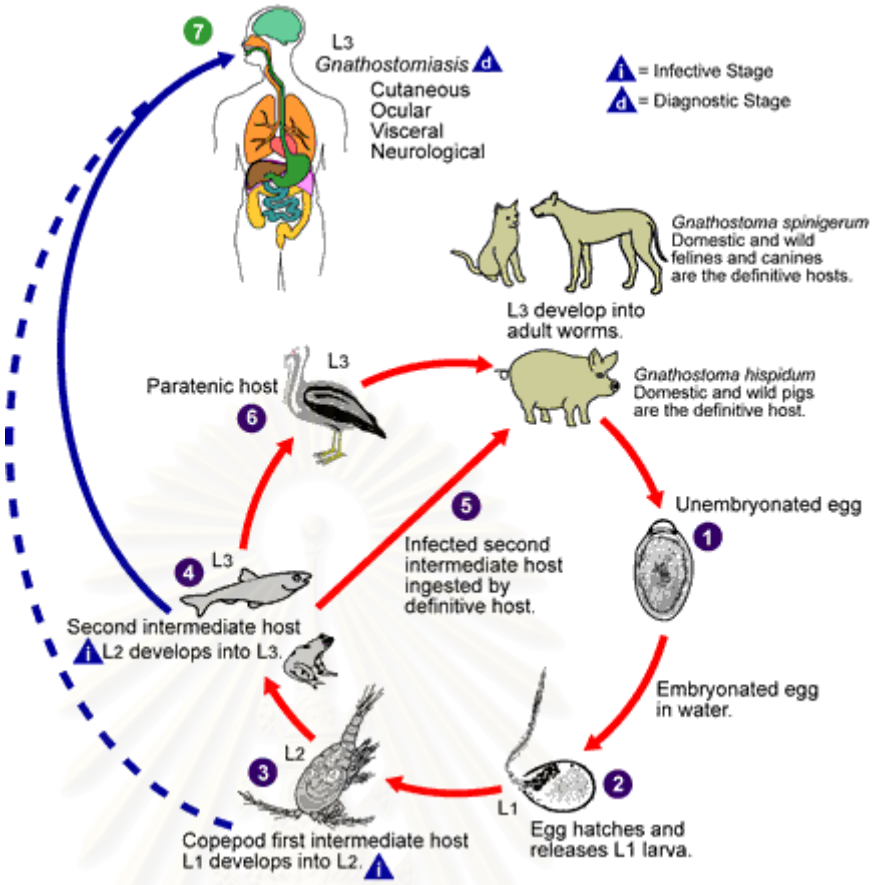


Fig 1. Life cycle of *Gnathostoma spinigerum*

1.4 Morphology

The parasite has a characteristic head-bulb providing with cephalic hooklets. The number of hooklets generally varies from one row to another. The morphology and the arrangement of hooklets are used for the identification of species (Daengsvang, 1980). The anterior half of the body is covered with transverse rows of flat, toothed spines, followed by a bare portion. The posterior tip of the body has numerous tiny cuticular spines.

Adults worm, males average measures are 12-40 mm x 1.0-3.0 mm and have a bluntly rounded posterior end. Female average measures are 25-54 mm x 1.0-3.0 mm and also have a blunt posterior end.

The egg is 65-70 µm x 38-40 µm and colorless. Its polar cap is at only one end.

Advanced L3s possess a typical head-bulb armed with 4 rows of cephalic hooklets and a pair of trilobed lips. Each row has about 40 or more in number, which increase posteriorly. The average size is 5.0 mm long, but can up to 12.5 mm.

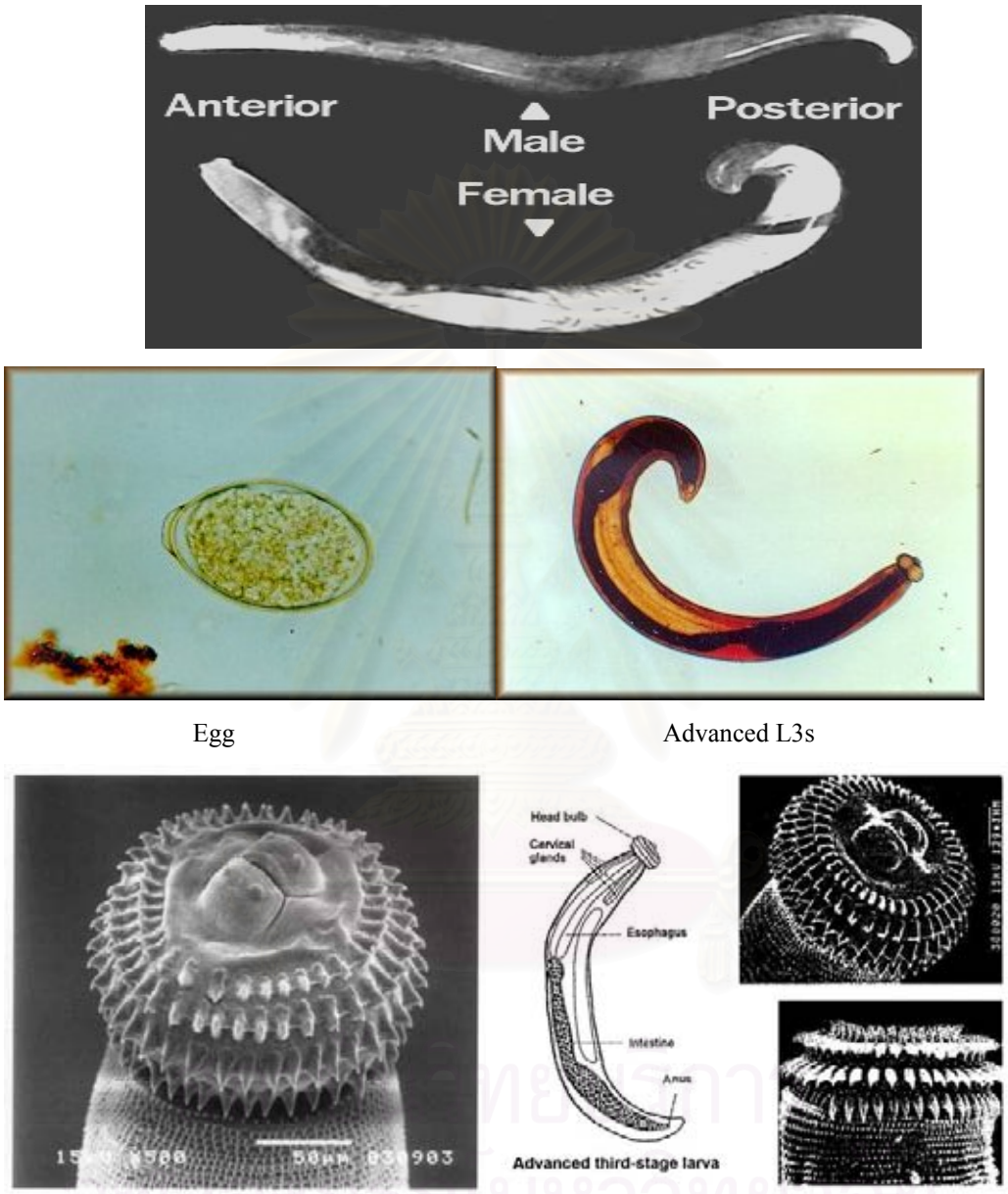


Fig 2. Morphology of eggs, advanced L3s (Head-bulk, hooklets) and adult *G.spinigerum*

1.5 Modes of transmission

Experimentally, there are 3 following methods for spreading the infection:

1.5.1 Oral transmission

The major route of infection is ingestion of uncooked larvae presented in fresh water fishes that harbor the infective stage.

1.5.2 Skin penetration

The infective stage or the advanced L3s is capable of penetrating the skin of the host (Prommas and Daengsvang, 1933).

1.5.3 Prenatal infection

Prenatal transmission of advanced L3s from mother to offspring can occur in experimental mice. In man, two cases of prenatal gnathostomiasis were reported. It was the three-day old baby, which had creeping eruption with immature male on the skin of the chest. Another was one month old baby with immature worm removed from the skin of the umbilical area (Daengsvang, 1986).

2. Clinical manifestations and pathology

During 1961 and 1963, about 900 patients with gnathostomiasis were clinically diagnosed each year in Thailand. There are about 10 deaths reported between 1967-1981 (Daengsvang, 1980; Daengsvang, 1986). In each year from 1985 to 1988, between 300-600 suspected gnathostomiasis patients came to the gnathostomiasis clinic at the hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University, Bangkok. Although in the recent past, people have received better health education, the disease still prevalent, possibly because of their social customs and dietary habits (Tuntipopipat, 1989). From 1989 until now, about 100-400 new cases of suspected gnathostomiasis have been seen each year at this hospital (Rojekittikhun, 2002). At Chulalongkorn Memorial Hospital in Bangkok, approximately 100-150 patients of the new suspected cases come to the Parasitology Clinic each year (Kraivichian, 2004).

2.1 Symptoms and pathogenesis

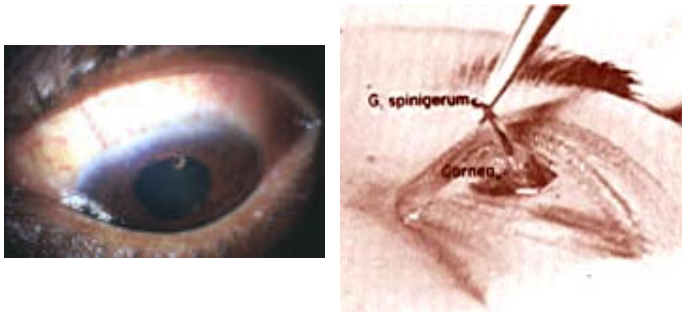
A few days after advanced L3s are ingested, these larvae will migrate through the intestinal wall and into the abdominal cavity and then through several tissues causing lesion in many organs and subcutaneous tissues. Mechanical action associated with migrating worm and host responses may also contribute to two

classified pathological changes: namely cutaneous and visceral gnathostomiasis (Miyazaki, 1960).

For cutaneous gnathostomiasis, it appears as intermittent migratory swelling of various circumscribed sizes lasting one to two weeks. The edema is mainly due to the inflammatory reaction of the tissue with mononuclear cell, mainly eosinophil infiltration (Boongird et al., 1977; Rusnak et al., 1993; Kraivichian et al., 2004). Patients can have allergic reaction with itching or irritation with pain at the swelling areas.

For visceral gnathostomiasis, L3s may migrate to the central nervous system (CNS) (Chitanondh and Rosen, 1967; Punyagupta, 1968). When cerebral gnathostomiasis occurs, the disease is known as eosinophilic myeloencephalitis with long-term sequel or even death. The CSF pressure in these patients is high with a marked increase of white blood cells, especially eosinophils. The clinical course of CNS gnathostomiasis that involves only the spinal cord leads to paralysis of extremities (Bunnag, 1970). A severe agonizing pain over the trunk and lower extremities commonly appears before paralysis occurs (Punyagupta, 1968). The mortality of CNS gnathostomiasis was reported to be as high as 25 percent (Punyagupta, 1978). Gnathostomiasis is one of the three most common parasitic infection that involve the CNS in Thailand (Vejjajira, 1978; Tuntipopipat et al., 1989).

a



b

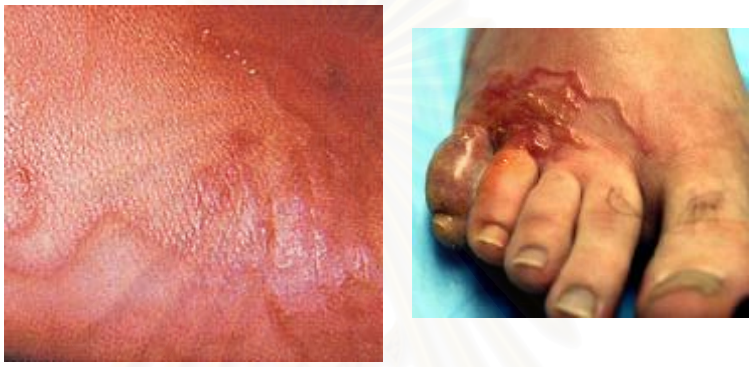


Fig 3. Symptoms of human gnathostomiasis

a: *G.spinigerum* from eyeball

b: creeping eruption

2.2 Diagnosis

The definitive diagnosis of human gnathostomiasis must be made by recovery of the worm. However, if the parasite is not recovered the following clinical and laboratory findings of the suspected cases may be useful in bringing closer diagnosis to the infection:

1) Presumptive diagnosis may be made on the basic of clinical symptoms and signs for cutaneous and visceral gnathostomiasis.
2) Previous history of eating raw or improperly cooked fish.
3) History of living in or visiting the endemic or near-by area of the infection.
4) Blood picture

Gnathostomiasis should be suspected when a localized edema is accompanied by leucocytosis with a high percentage of eosinophils. Leucocytosis of

over 10,000 cells/mm^3 is common and between 10 and 96 percent of the cells are usually eosinophils (Daengsvang, 1980; Nuchprayoon et al., 2003; Kraivichian et al., 2004).

5) Immunodiagnosis

Since, there is similarity between the syndromes caused by other parasitic infections such as angiostrongyliasis and many ectopic parasitic infections and that of gnathostomiasis; the miss diagnosis may be made (Tuntipopipat et al., 1989; Tapchaisri et al 1991). Following were the immunological test for detecting antibodies.

a) Skin test

The skin test was firstly used to diagnose human gnathostomiasis, it gave 100% sensitivity but poor specificity (Egashira, 1953 and Yamaguchi, 1951; Chanthachume et al., 1985). The extract of the esophagus was more antigenic than that of the other parts of the worm (Miyazaki, 1960; Miyazaki, 1966). In experimental animal, this skin reaction could be demonstrated at the first month of infected mice (Morakote et al., 1987).

b) Precipitin test

A precipitation reaction appeared prominently in the anterior tip of the adult worm within 2 weeks and reached a peak at the 6^{th} week and declined within 10 to 18 weeks (Furuno, 1959).

Microprecipitation reaction of larva with infected human and experimental animals sera showed membranous precipitates around esophageal region and filamentous precipitates randomly on the surface of the worm. This indicated that the epitopes are located exteriorly on the advanced L3s. (Rojekittkhun, 1989).

c) Indirect hemagglutination antibody test (IHA)

Haemagglutinating (HA) antibodies reactive with advanced L3s somatic extract could be detected as early as one week after infection with titer 1:40 (Priwan, 1985). The titer gradually increased to 1:1,280 within eight weeks.

d) Enzyme Linked Immunosorbent Assay (ELISA)

By using advanced L3s *G.spinigerum* as an antigen, IgG ELISA has been trialled to diagnose human gnathostomiasis. The test showed 100% positive in all parasitological confirmed cases and all eosinophilic meningo-myeloencephalitis cases. But not all of the sera from cutaneous migratory swelling cases (CMS) were positive (Suntharasamai et al., 1985; Dharmkrong-at et al., 1986).

Similar results were obtained when excretory and secretory (ES) antigen which released in the culture medium with the highest amount at day 4 was used (Rattanasiriwilai et al., 1985). However, these ELISA showed superior results in diagnosis than that of indirect hemagglutination test and counter immunoelectrophoresis (Maleewong et al., 1988).

In addition, the total IgE and specific IgE in gnathostomiasis sera were markedly higher than healthy controls. This indicated that *G.spinigerum* potentiates IgE production (Soesatyo et al., 1987 and Saksirisampant et al., 2001).

Isotype of both IgG and IgE cross-react with following infections namely: angiostrongyliasis, opisthorchiasis, filariasis, paragonimiasis, strongyliasis, enterobiasis and hook worm infection (Suntharasamai et al., 1985; Soesatyo et al., 1987; Maleewong et al., 1988; Saksirisampant et al., 2001).

The use of IgG subclass antibodies could improve the overall sensitivity and specificity of the anti-*G.spinigerun* L3s ELISA. The detection of IgG2 antibody against *G.spinigerum* L3s reduced cross reactivity to most parasites, particularly *A.cantonensis*. The ELISA IgG1 antibody was suggested a reliable laboratory screening test, while anti-*G.spinigerum* L3s IgG2 antibody could be used to confirm the diagnosis (Nuchprayoon et al., 2003).

Since IgG-Ab could persist more than six months following drug treatment (Kraivichian et al., 1992 and Nontasut et al., 2000); thus the evaluation of the effectiveness of chemotherapy by detecting this IgG-Ab was still not satisfactory. However, suitable immunodiagnosis for such evaluation has not been reported.

By using a two-site enzyme-linked immunosorbent assay, the circulating antigen was detected at the first week, and increased steadily up to week 3, with the peak at week 4 of infection. There was no significant amount of the circulating antigen which was detected after week 4 of infection (Maleewong et al., 1992).

e) Western blot analysis

About 20 protein components of advanced L3s *G.spinigerum* has been shown by SDS-PAGE with relative molecular weight ranging from 10 to 99 kDa (Priwan, 1985; Saksirisampant, 1986). This purified 24 kDa protein demonstrated 100% specificity and sensitivity in ELISA when tested with parasitologically and clinically diagnosed gnathostomiasis patients (Nopparatana et al., 1991). However, from many study, it has been shown that specific antigen of *G.spinigerum* is a protein with molecular weight of 24 kDa, while bands of 43 and 49 kDa showed

reaction with IgE-Ab (Tapchaisri et al., 1991; Saksirisampant, personal communication). The protein band of approximately 50 kDa showed specificity to mice infected sera as well (Priwan, 1985).

2.3 Treatment

In the past, the success rate of thiabendazole, praziquantel, metronidazole, diethyl carbamazine and quinine in either experimental animals or in human, was unsatisfactory (Yingyourd and Daengsvang, 1983; Yingyourd et al., 1989; Setasuban et al., 1981). At the present time, the broad spectrum anthelmintic used in clinics and in this study will be detailed below.

1) Albendazole

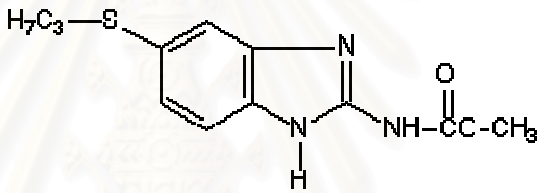


Fig 4. Structure of albendazole

Albendazole (Zentel^{R}), is a benzimidazole derivative (methyl-5-propylthio-1-H benzimidazole-2yl carbamate).

This drug interferes with normal metabolic function and block glucose uptake by adult intestinal-dwelling helminthes and their tissue-dwelling larvae. This caused a decrease in the formation of ATP, which is essential to the survival and reproduction of the helminthes (Bennett and Guyatt, 2000). Its parasiticidal activity was shown at a high dose of 200 mg/kg for 21 consecutive days in experimental infected mice (Yingyourd et al., 1985).

In terms of treatment, clinical cure of cutaneous gnathostomiasis is not defined only by the disappearance of the cutaneous lesion. Disappearance of cutaneous swelling was found in both the albendazole treatment and placebo groups with a similar duration (mean duration = 6.8 days). It is possible that the swellings disappeared following migration to deeper tissues (Kraivichian et al., 1992).

Therefore, serologic tests and absolute eosinophil counts were also indicators of clinical cure. An animal study showed that 2-4 weeks after infection, rats containing advanced L3s showed elevated peripheral eosinophil counts that gradually returned to normal after five weeks because the larvae were encysted in rat muscles and thus caused no eosinophilic stimulation (Ogata et al., 1998).

In case of human gnathostomiasis, a dose of 400 mg/kg body weight once daily for 21 days has also been reported, with cure rate of 94.1% (Chitchang, 1987, Kraivichian et al., 1992; Inkatanuvat, 1998). However, the gastrointestinal distress, headache, dizziness, increasing and reversible levels of hepatic enzymes, and transient reduction of the total leucocyte count have been its side effects.

2) Ivermectin

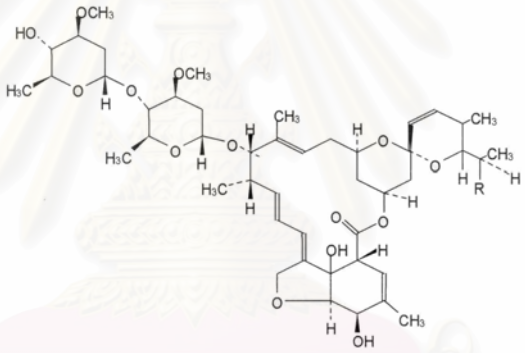


Fig 5. Structure of ivermectin

Ivermectin (Ivermec^{R}), the 22,23-dihydroavermectin B1 derivative of avermectin B, is a semisynthetic macrocyclic lactone derived from the actinomycete *Streptomyces avermitilis*.

Ivermectin stimulates the release of gamma aminobutyric acid (GABA) from the nerve endings and enhances binding of GABA to its receptor on the postsynaptic membrane of the motor neurons by combining with some other part of the GABA-receptor-ionophore complex. This results in hyperpolarization, blocking of neuromuscular transmission and paralysis of the worm (Campbell, 1985).

It has been shown to affected to *Onchocercus spp*, *Trichuris trichiura*, *Strongylus spp* and filarial worm (Duke et al., 1990; Contiho et al., 1994; Marti et

al., 1996). A single dose (150-200 μg/kg) of ivermectin is also highly effective in the treatment of cutaneous larva migrants (creeping eruption), with cure rates of 100%. Its side effects were hypotention, dizziness, weakness and diuresis (Nontasut, 2000).

At a single dose of 0.2 and 2 mg/kg body weight of ivermectin subcutaneously, yielded a reduction of larvae of *G. spinigerum* 74.2% and 84.2%, respectively (Anantaphruti et al.,1992). The dose of 0.2 mg/kg for 5 days which was orally given to rat gnathostomiasis demonstrated 87.9% of worm reduction and 25% of cure rate (Waikagul et al., 1994).

Ivermectin had mild side effects and was more convenient use to single dose than that of albendazole (Nontasut, 2000). The drug trial of ivermectin in human gnathostomiasis showed 76% cure rate using 0.2 mg/kg single oral dose. While albendazole gave 92% cure rate using 400 mg/day for 21 day. Although different cure rates were not statistically significant, ivermectin seemed to be a better drug of choice for treatment of human gnathostomiasis (Kraivichian, 2004).

3. Immunological studies on Gnathostomiasis

3.1 Antigenicity of *Gnathostoma spinigerum*

Nematode has a wide variety of antigens capable of stimulating immune responses in mammalian hosts, namely: surface or cuticular, metabolic or excretory-secretory (ES) and somatic antigens. Moreover, all of these antigens can be found in all developmental stages, starting from larval to mature adult. The surface components can be prepared by solubilizing the parasites with either ionic or non-ionic detergents. Excretory-secretory products can be obtained from an in vitro maintenance or cultivation of living parasites in protein-free culture medium. Generally, the most common source is the whole worm or somatic extract.

The larvae stage antigen of *G.spinigerum* which migrant in host is more immunogenic than that of adult worm. The sensitivity of larvae somatic antigen by ELISA was 100% while that of adult female and male somatic antigens were 70%, 60% respectively (Morakote et al., 1991).

Western blot analysis has been shown that a protein with a relative molecular weight of 24 kDa prepared from the advanced L3s is highly specific to this infection (Tapchaisri et al., 1991). Among the three preparation of advanced L3s somatic extract from distilled water, Triton X-100 and sodium deoxycholate (NaDOC), the distilled water preparation yielded the greatest amount of diagnostic

24 kDa antigen. Little difference in protein and antigenic patterns was found between the extracts of larvae collected from naturally infected eels and from experimentally infected mice (Nopparatana et al., 1992). This 24 kDa component was found predominantly in the esophagus and intestine. By in direct fluorescent antibody assays the target antigens were anterior part of the esophagus, surface of the cuticle and in the cytoplasmic granules of the intestine (Morakote et al., 1989).

The ES products may be present more potential components than those of the somatic extract for evaluation of the effectiveness of chemotherapy. Because the antibody responses to secreted products are more closely related to active infection than the anti-whole worm antibody that may persist following the death of the parasites (Saksirisampant et al., 2001).

3.2 Antibody responses

IgG and IgE antibodies response were induced in gnathostoma infection. The sensitivity of ELISA for these anti-*G.spinigerum* antibodies ranged from 59% to 95%, while the specificity ranged from 79% to 96% (Suntharasamai et al., 1985; Maleewong et al 1988; Anantaphruti, 1989; Saksirisampant et al., 2001).

In addition, the IgG antibody had quite persisting titers in post-treatment condition of many parasitic infections including gnathostomiasis (Nontasut et al., 2000). IgG antibody persist for several months after successful treatment. These facts should be taken into consideration in the interpretation of serological results, since positive IgG antibody level may reflect a previous rather than a present infection. In many helminthic infections, the activated Th-2 cells secrete IL-4 and IL-5, which stimulates the production of IgG1, IgG4, IgE and eosinophils. In *G.spinigerum*, evidences previously showed that gnathostomiasis sera had an elevation of IgG1, IgG4 and IgE.

3.3 Cellular immune responses

In contrast to the antibody response, cell-mediated immune response to this nematode infection has not been reported.

It is well recognized that various types of infections, including protozoa and helminthes may be associated with alteration of immune response to specific antigens and mitogens. Both immunoenhancement and immunodepression have been reported in many host-parasite systems. And most nematode infections are chronic

in nature because of their ability to evade or resist elimination by adaptive immunity (Terry and Hunson, 1982).

Parasites evade protective immunity by reducing their immunogenicity and by inhibiting host immune responses. Different parasites have developed remarkably multiple mechanisms of resisting immunity such as antigenic variation, inhibition of host immune responses and antigen shedding. T-cell anergy to parasite antigens has been observed in severe schistosomiasis involving the liver and spleen and in filarial infections. In lymphatic filariasis, infection of lymph nodes with subsequent architectural T-cell disruption may contribute to deficient immunity. In malaria and African trypanosomiasis generalized immunosuppression is caused by the production of immunosupressive cytokines from activated macrophages and T cells (Abbas and Lichman, 2003).

The immunodepression which has been demonstrated in several helminth infections was more obvious in those of heavy and chronic infection. For example, hamsters experimentally infected with *Opisthorchis viverrini* had significant depression of lymphoproliferative response to mitogen, homologous and heterlogous antigens. These immune-alteration was abolished by anthelmintic treatment. It was suggest that defective T cell functions could interfere immune surveillance system against neoplastic transformation. Since, chlolangiocarcinoma was the severe complication of this liver fluke infection (Sirisinha et al., 1983). Furthermore, in schistosomiasis the defective T-cell function demonstrated by lymphocyte proliferation resulted in drastically decreased of egg granulomatous reaction. T-cell defect may be considered as a host evasion of the parasite and as a modulation of the host immune reaction. Since adaptive T-cell responses to parasites can contribute to tissue injury. The severity of the immune reaction or the hypersensitivity can be depressed (Abbas and Lichman, 2003).

Study of T-cell function or adaptive T-cell response in animal infected with this parasite may lead to the understanding the host-parasite relationship in term of immunity and pathophysiology.

CHAPTER IV

MATERIALS AND METHODS

1. Experimental infection of mouse with *Gnathostoma spinigerum*

1.1 Animals

Eight weeks old male outbred ICR strain mice, weighed 34±2 grams, were purchased by the National Laboratory Animal Center, Salaya campus, Mahidol University. The mouse was highly susceptible to and easily infected with *G.spinigerum*. They were reared strict hygienic conventional in polycarbonate shoe-box, in the air conditioned room with a constant temperature of approximately 22-25 ^{o}C in the animal house, Faculty of Science, Mahidol University and were fed ad libitum with food pellet and dechlorinated tap water. The behavior and physiological changes of mice such as social interactions, feeding, drinking, sleeping, wool and tears were observed twice a week.

1.2 Collection of advanced third stage larvae (L3s)

The visceral organs of the infected swamp eels (*Monopterus albus*) were purchased from Klong Toey and Tevej markets in Bangkok. The livers were separated, washed 2-3 times with tap water and were then cut into small pieces. Approximately, 1 part of the small cut livers was digested in 3-4 parts of 1.5% (w/v) pepsin solution (Sigma, U.S.A.) pH 2.0 for 4 hours at 37 ^{o}C. Thereafter the digested tissue was put into a conical flask. The settle sediment was washed by resuspending in normal saline solution (0.85% NaCl). The washing and sedimentation was repeated twice more and then the final sediment was examined under a dissecting microscope. The L3s were individually picked from the digested liver. The larvae were identified to species as described by Daengsvang, 1980.

1.3 Preparation of crude somatic L3s Antigen

The L3s were homogenized in a ground glass tissue grinder with a small volume of sterile distilled water. The ground-up worm was then sonicated under an Ultrasonic Disintegrator (Soniprep 150, MSE Scientific Inc., England) at an

amplitude of 6 microns at 1 min intervals. The sonication step was carried out until most cells were broken judging from microscopic examination. Thereafter it was centrifuged at 12,000 rpm for 20 min at 4^{o}C. The supernatant fluid was obtained. Protein content of the extract was determined by the Lowry's method using bovine serum albumin (BSA, crystalline form, Sigma, U.S.A.) as a standard. The crude somatic L3s extract was kept in small aliquots at -70^{o}C.

1.4 Infection of mouse with advanced third stage larvae of *G. spinigerum*

Each animal was infected with L3s *G.spinigerum* through 5 ml syringe attached to polyethylene gastric tube. The animal was held tightly without anesthesia and the tube was gently passed through the oral cavity into the esophagus until reaching a stomach. Food and water were withheld for another 3-6 hours. Thereafter, pellet diet and water were given ad libitum.

The first and the second group of mice were infected with 10 larvae and 20 larvae of L3s. Half of the mice in each group was given with anthelmintic drug (ivermectin) ten days after.

2. Treatment of infected mouse with Ivermectin

Ivermectin in form of solution (with molecular weights of 872.3) was obtained as the commercial preparations Ivomec (MSD AGVET, Hoddesdon, U.K.) containing 1%(w/v) sterile solution of drug. A single oral dose of ivermectin at 0.4 mg/kg body weight was given to each animal.

3. Source of normal sera and anti-*G.spinigerum* sera

Blood was collected from the opthalmic venous plexus of normal and infected mice by using microhematocrit tube as described by Goligen and Shevach, 1994. Sera were separated and kept at -20 ^{o}C.

4. Antibody determination by Enzyme Linked Immunosorbent Assay(ELISA)

The indirect technique of enzyme-linked immunosorbent assay (ELISA) of Tuntipopipat et al. (Tuntipopipat et al., 1993) was followed in this study.

4.1 Optimizing condition for ELISA

The optimal concentration of antigen for coating the wells of microtiter plates was determined by performing checkerboard titrations with two-fold dilution of antigen starting from 1.25 to 5 μg/ml and five-fold serial dilutions of both positive and negative sera starting from 1:25 to 1:625. The optimal concentration was 5 μg/ml.

4.2 Determination of serum antibody specific for *G.spinigerum*

The antigen was diluted with coating buffer (0.05 M Carbonate) pH 9.6, to a concentration of 5 μg/ml. Fifty microliters of the antigen solution was added to each well of 96-well flat-bottomed microplate (Maxisorp-Nunc, U.S.A.). After an overnight incubation at 4^{o}C, the plates were washed three times by using immuno washer (Nunc, U.S.A.). After tapping dry, the plates were then added with blocking buffer [1%Bovine serum albumin (BSA), Sigma, U.S.A.] for 60 min at 30^{o}C. The plates were washed again and tapped dry. Fifty microliters of serum were added to each well.

For determination of specific IgG antibody, each serum sample was diluted at 1:125 in 1% BSA buffer (buffer 10 ml +Tween 20 5 μl +0.1 gm BSA). The samples were then incubated for 60 min at 30^{o}C. After one more washing, fifty microlitres of 1:2,000 dilution of horseraddish peroxidase (HRP)-conjugated rat anti-mouse immunoglobulin G was in PBST-1%BSA was added to each well. After three times washing, one hundred microlitres of substrate 3,3′,5,5′-tetramethyl benzidine (TMB, 0.1 mg/ml) was added. The plates were then incubated at the dark at room temperature for 10 min, and the reaction was terminated by the addition of 25 μl of 5N sulfuric acid.

For determination of specific IgG subclass 1 and subclass 2a antibody, the procedures are the same as above protocol of IgG-Ab. Differences of the procedure are the dilution of serum, enzyme conjugated anti-immunoglobulin, substrate and incubation time. Each serum are similar to sample was diluted at 1:125 and dilution used for HRP-conjugated rabbit anti-mouse immunoglobulin G1 was 1:2,000 and HRP-conjugated rabbit anti-mouse immunoglobulin G2a was 1:1,000 in PBST-1%BSA. Substrate was 3,3′,5,5′-tetramethyl benzidine (TMB, 0.1 mg/ml) in 0.1 M acetate buffer pH 6.0 containing 0.0035% H_2O_2. The incubation time was 10 min.

For determination of specific IgE antibody, the procedures are the same as above protocol of IgG subclass 1. Differences of the procedure are dilution of serum, and the enzyme conjugated anti-immunoglobulin and incubation time. Each serum sample was diluted at 1:25 and samples were incubated for 120 min at 30^{o}C. The dilution used for rat anti-mouse immunoglobulin E was 1:400 and HRP-rabbit anti-rat immunoglobulin G was 1:2,000 in PBST-1%BSA.

The absorbance of the yellow color developed with substrate 3,3′,5,5′-tetramethyl benzidine (TMB) was read at 450 nm with a Minireader II Photometer (Dynatech Laboratories, U.S.A.).

5. Lymphoproliferation assay

Lymphoproliferative responses of spleen cells stimulated by Concanavalin A (Con A) and crude somatic antigen of L3s *G.spinigerum* were studied. The rate of DNA synthesis following mitogen and antigen stimulation was determined by the in corporation of tritiated thymidine (^{3}HT). Sterile microcultured technique was modified from one described by Kalra et al., 2002 and Sciutto et al., 1995 and was performed in a laminar airflow controlling equipment (Nuaire, Canada).

5.1 Culture medium

Roswell Park Memorial Institute medium 1640 (RPMI 1640, Gibco, U.S.A.) was used to culture the lymphocytes (see appendix).

5.1.1 Working medium I

RPMI 1640 powders were dissolved in deionized distilled water. Penicillin G (Sodium penicillin G, Merck), streptomycin (Streptomycin sulfate B.P., Glaxo) and HEPES (N-2-hydroxyethylpiperazine N-2-ethanesulfonic acid, MW 238.3, Sigma, U.S.A.) were added to final concentration of 100 units/ml, 100 μg/ml and 25mM respectively. Before used, pH of the medium was adjusted to 7.2-7.4 with 7.5% $NaHCO_3$. The working medium I solution was then sterilized by membrane filtration (Millipore, 0.22 μm pore size) and could be kept at 4^{o}C.

5.1.2 Working medium II

The working medium I was supplemented with 10% (v/v) fetal bovine serum (Gibco, U.S.A.), 4 mM L-glutamine (Gibco, U.S.A.) and 50 μM 2-mercaptoethanol. The working culture medium II was made up freshly each time.

5.2 Preparation of Concanavalin A

Five hundred milligrams of Concanavalin A (Sigma, U.S.A.) was dissolved in 5 ml of sterile PBS, divided into small aliquots and kept frozen at -20 ^{o}C. The concentration of the stock solution was 1 mg/ml. The stock solution was thawed only once diluted with working RPMI 1640 medium II to the required concentration of 1 μg/ml and used immediately.

5.3 L3s *Gnathostoma spinigerum* somatic antigen

The crude somatic antigen of L3s was thawed and diluted with working RPMI 1640 medium II to a concentration of 100 μg/ml and steriled by membrane filtration (millipore 0.45 μm pore size).

5.4 Splenic cell suspension

Normal and infected mice were sacrificed by anesthesia with ether in fume hood. Spleens were removed aseptically and placed in sterile petridish containing working RPMI 1640 medium I. Single cell suspension was prepared by gently pressing the spleen through a sterile stainless steel sieve. The spleen cell suspension was pipetted up and down 5 times to make a single cell suspension. The cells were washed 2 times with working RPMI 1640 medium I in a 15 ml polypropylene conical tube (Costar, U.S.A.), followed by centrifugation at 1,500 rpm for 5 minutes at room temperature. Spleen cells were resuspend with 1 ml working RPMI 1640 medium II for cell culture. After adding the trypan blue dye, spleen cells were counted in a hemocytometer. The suspension was adjusted to contain $5x10^6$ cells/ml with working RPMI 1640 medium II.

5.5 Method for cell culture

One hundred microlitres of 5 x 10^6 spleen cells/ml were added in triplicate wells of 96-well flat bottom plastic tissue culture plate (Costar, Corning, NY, U.S.A.). One hundred microlitres of crude somatic antigen (100 μg/ml) or Concavalin A (1 μg/ml) were added to the well with cells. The final volume of the medium in each well was 200 μl. The final concentration of Con A in the well was 0.5 μg/ml and that of antigen was 50 μg/ml. The final concentrations of serum, L-glutamine and 2-mercaptoethanol in the cell culture medium were 10%, 4mM and

50 μM respectively. The culture plate was incubated at 37 ^{o}C in a humidified incubator with 5% CO_2 atmosphere (Forma Scientific, U.S.A.).

5.6 Determination of thymidine incorporation

For mitogen stimulation, the cells were cultured 48 hours before pulsing with 50 μl of 20 μCi/ml of tritiated thymidine (1 μCi/well, M.W.242, Amersham). The cultures were incubated further under the same condition for another 18 hr before the cell were harvested. On the other hand, when the cell were stimulated with the antigen they were cultured for 5 days and pulsed with ^{3}HT as described for the mitogen. After 18 hr incubation, and cells were harvested in the multiwell harvestor (Nunc, U.S.A.) and rinsed with distilled water on a glass fiber filter paper (Whatman, England). The dried filter discs were placed in glass scintillation vials. One milliliters of scintillation fluid containing 0.5% (W/V) PPO (2,5-Diphenyloxazole, Eastman) and 0.1%(W/V) POPOP (5-phenyloxazole, Eastman) in toluene was added to each vial. All vials were pre cooled in the dark for at least 3 hours before counting. Radioactivity was counted (5 min) in a liquid scintillation β-counter (Beckman, U.S.A.).

5.7 Data analysis

5.7.1 Change in counts per minute (S-U or Δ cpm)

The value represented the absolute difference in cpm between the stimulation cultures (S) and the corresponding unstimulated controls (U).

Δ cpm = mean cpm of stimulated culture – mean cpm of unstimulated culture

5.7.2 Stimulation index (SI)

$$\text{S.I.} = \frac{\text{mean cpm of stimulated culture}}{\text{mean cpm of unstimulated culture}}$$

5.7.3 Statistical test

One-Way ANOVA and Student's t test were used.

CHAPTER V

RESULTS

1. Antibody response of mice infected with *G.spinigerum*

1.1 Standardization of ELISA

1.1.1 Optimization of specific IgG antibody

Pooled sera obtained from mice infected with 20 larvae of *G.spinigerum* at day 50 and pooled normal sera collected from normal healthy mice were used for the standardization assay. The optimal concentration of advanced L3s antigen was determined by performing a checkerboard titration with two-fold dilution of antigen starting from 1.25 to 5 μg/ml. The normal control serum samples serially diluted five-fold from 1:25 to 1:625. The most optimal binding of specific IgG antibody was obtained with an antigen concentration at 5 μg/ml. It was therefore decided that, advanced L3s at a concentration of 5 μg/ml and dilution of serum samples at 1:125 was used for IgG determinations.

The optimal condition for the horseradish peroxidase (HRP)-conjugated rat anti-mouse IgG was determined by testing the conjugate diluted at 1:1,000, 1:1,500 and 1:2,000. The optimal dilution for this conjugate was 1:2,000. Enzymatic reaction with substrate of 3,3′,5,5′-tetramethyl benzidine (TMB) was allowed to take place for 10 min (Fig 6).

1.1.2 Optimization of specific IgG subclass 1 antibody

A similar protocol of infected and normal control sera was performed as described for that of IgG antibody. The antigen diluted to a concentration of 5 μg/ml and five-fold serial dilutions of both positive and negative sera starting from 1:25 to 1:625 were used. The most optimal binding of specific IgG subclass 1 antibody was obtained with serum sample diluted at 1:125.

The optimal condition for the HRP-conjugated rabbit anti-mouse Ig G1 was determined by testing the conjugate diluted two-fold from 1:1,000 to 1:4,000. The

highest absorbance reading was reached at 1:2,000. The enzymatic reaction was allowed to take place for 10 min (Fig 7).

1.1.3 Optimization of specific IgG subclass 2a antibody

Checkerboard titration was performed with two-fold dilution of antigen starting from 1.25 to 5 μg/ml and five-fold serial dilutions of both positive and negative sera starting from 1:25 to 1:625. The optimal binding of specific IgG subclass 2a antibody was obtained with an antigen concentration at 5 μg/ml and dilution of serum samples at 1:125.

The horseradish peroxidase (HRP)-conjugated rat anti-mouse IgG was diluted at 1:1,000 and 1:2,000. The optimal dilution for this conjugate was 1:1,000, whereas the enzymatic reaction was allowed to take place for 15 min (Fig 8).

1.1.4 Optimization of specific IgE antibody

The antigen was diluted to a concentration at 5 μg/ml and five-fold serial dilutions of both positive and negative sera starting from 1:5 to 1:25. The most optimal concentration of serum samples was 1:25.

The secondary anti-immunoglobulin E of rat anti-mouse IgE was tested with dilution of two-fold from 1:100 to 1:400. The enzyme conjugated of HRP-rabbit anti-rat immunoglobulin G was then added. The rat anti-mouse immunoglobulin E and HRP-rabbit anti-rat immunoglobulin G were used at dilution of 1:400 and 1:2,000 respectively, the enzymatic reaction was allowed to take place for 10 min (Fig 9).

All optimal components of ELISA of 4 antibodies were summarized in Table 1. The optimization of specific IgE antibody was different from the others. Because, two steps of enzyme-conjugated anti-immunoglobulin were used.

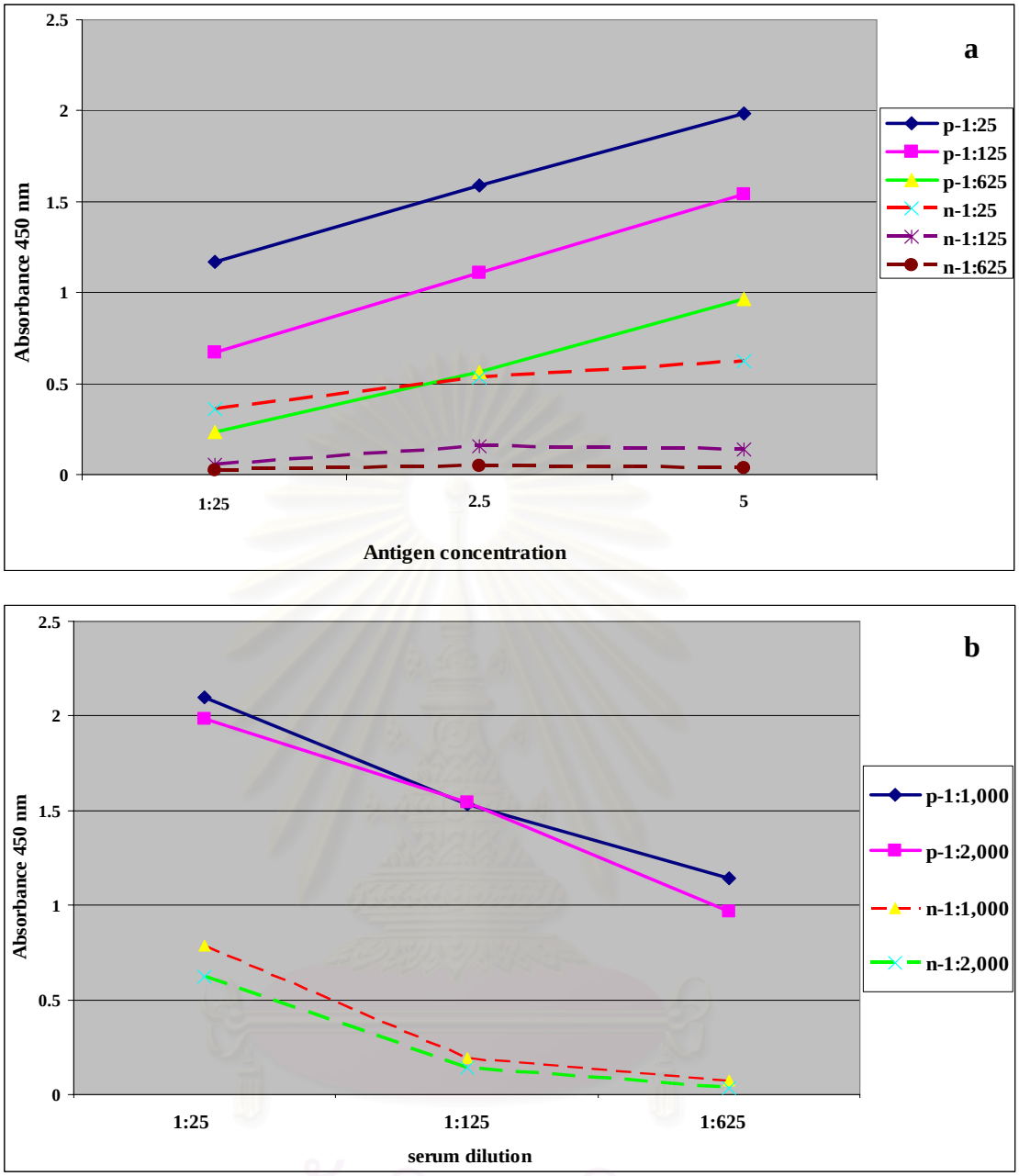


Fig 6. Checkerboard titration for IgG antibody against advanced L3s *G.spinigerum* antigen. Absorbance at 450 nm obtained by ELISA assays using pooled infected sera (P) and negative control sera (n) is shown using straight lines and dot lines, respectively.

a). Plates were coated with 50 µl of antigen at concentrations 1.25, 2.5 and 5 µg/ml. Serum dilutions used were 1:25, 1:125 and 1:625. Dilution of HRP-conjugated rat anti-mouse IgG was 1:2,000.

b). Plates were coated with 50 µl of antigen at concentration 5 µg/ml. Serum dilutions used were 1:25, 1:125 and 1:625. The dilutions of HRP-conjugated rat anti-mouse IgG were 1:1,000 and 1:2,000.

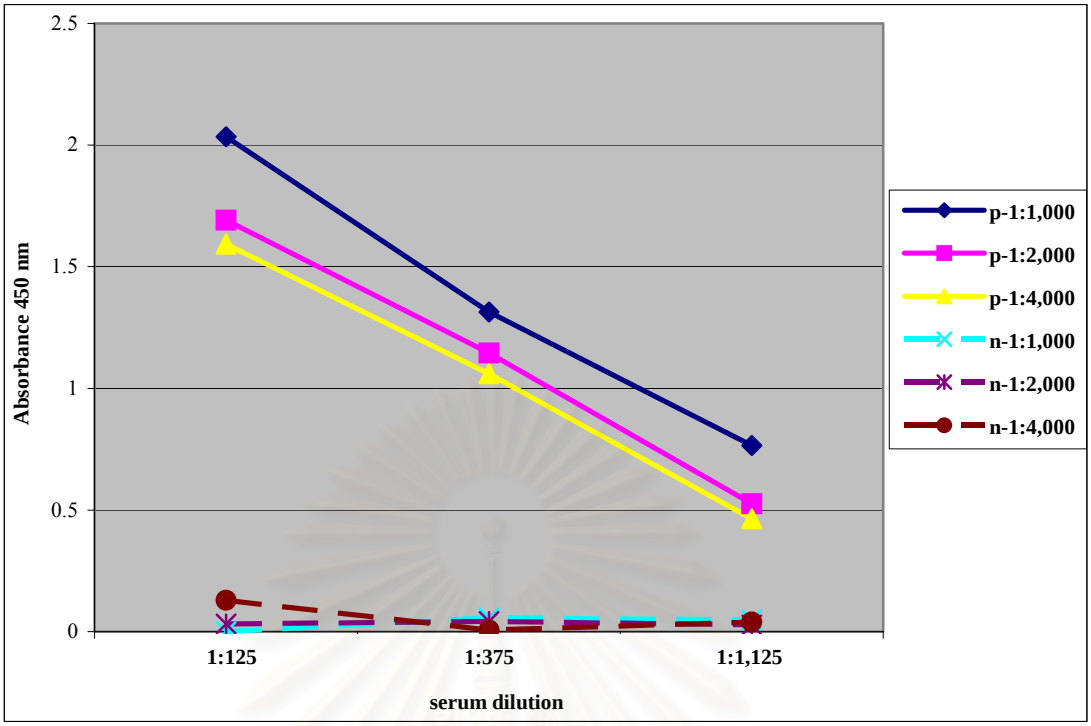


Fig 7. Checkerboard titration for IgG subclass 1 antibody against advanced L3s *G.spinigerum* antigen. Absorbance at 450 nm obtained by ELISA assays using pooled infected sera (P) and negative control sera (n) is shown using straight lines and dot lines, respectively. Plates were coated with 50 µl of antigen at concentration 5 µg/ml. Dilutions of sera used were 1:100, 1:300 and 1:900. Dilutions of HRP-conjugated rabbit anti-mouse IgG1 were 1:1,000, 1:2,000 and 1:4,000.

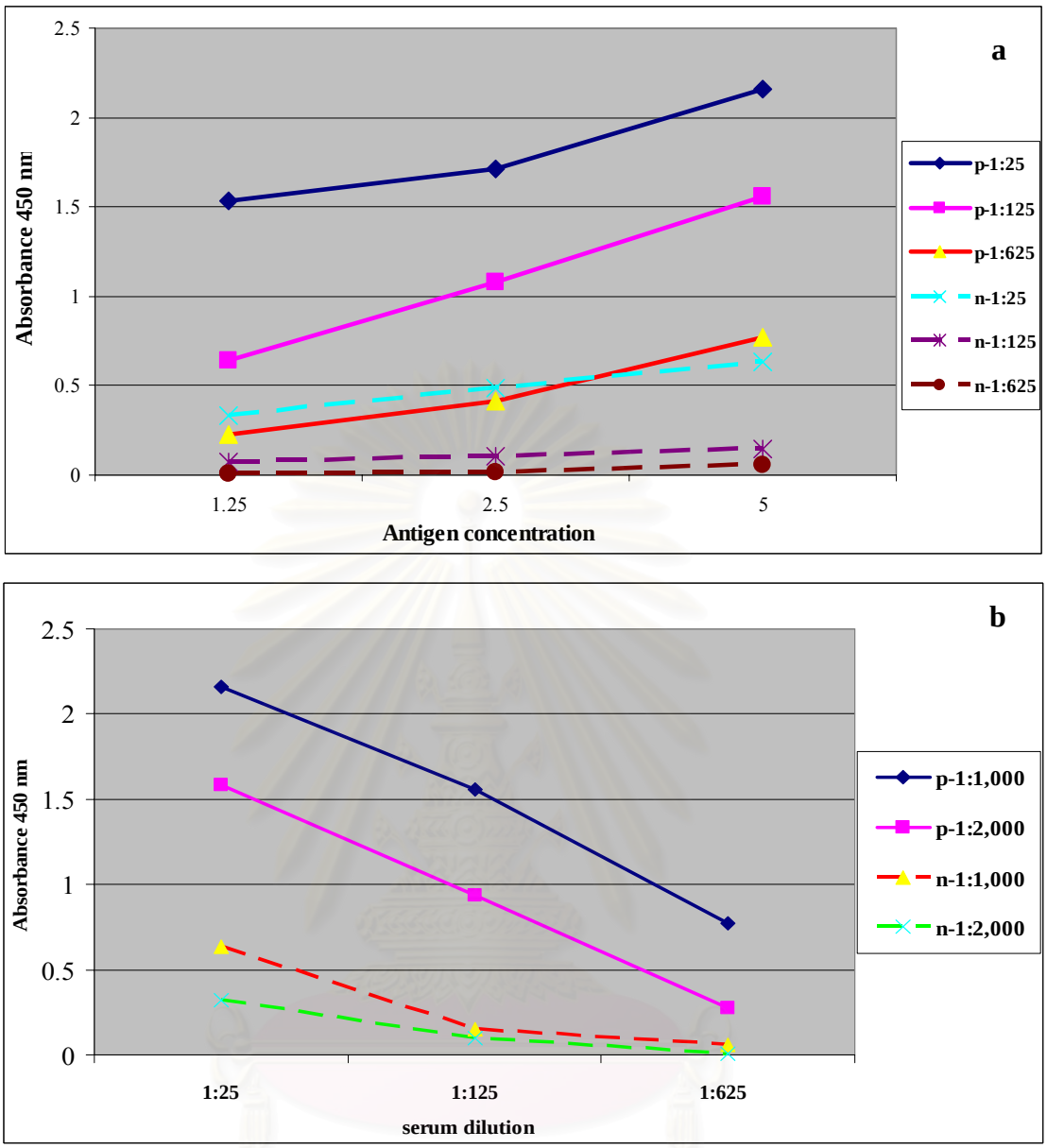


Fig 8. Checkerboard titration for IgG subclass 2a antibody against advanced L3s *G.spinigerum* antigen. Absorbance at 450 nm obtained by ELISA assays using pooled infected sera (P) and negative control sera (n) is shown using straight lines and dot lines, respectively.

a). Plates were coated with 50 µl of antigen at concentration 1.25, 2.5 and 5 µg/ml. Serum dilutions used were 1:25, 1:125 and 1:625. Dilution of HRP-conjugated rat anti-mouse IgG was 1:2,000.

b). Plates were coated with 50 µl of antigen at concentration 5 µg/ml. Serum dilutions used were 1:25, 1:125 and 1:625. The dilutions of HRP-conjugated rat anti-mouse IgG were 1:1,000 and 1:2,000.

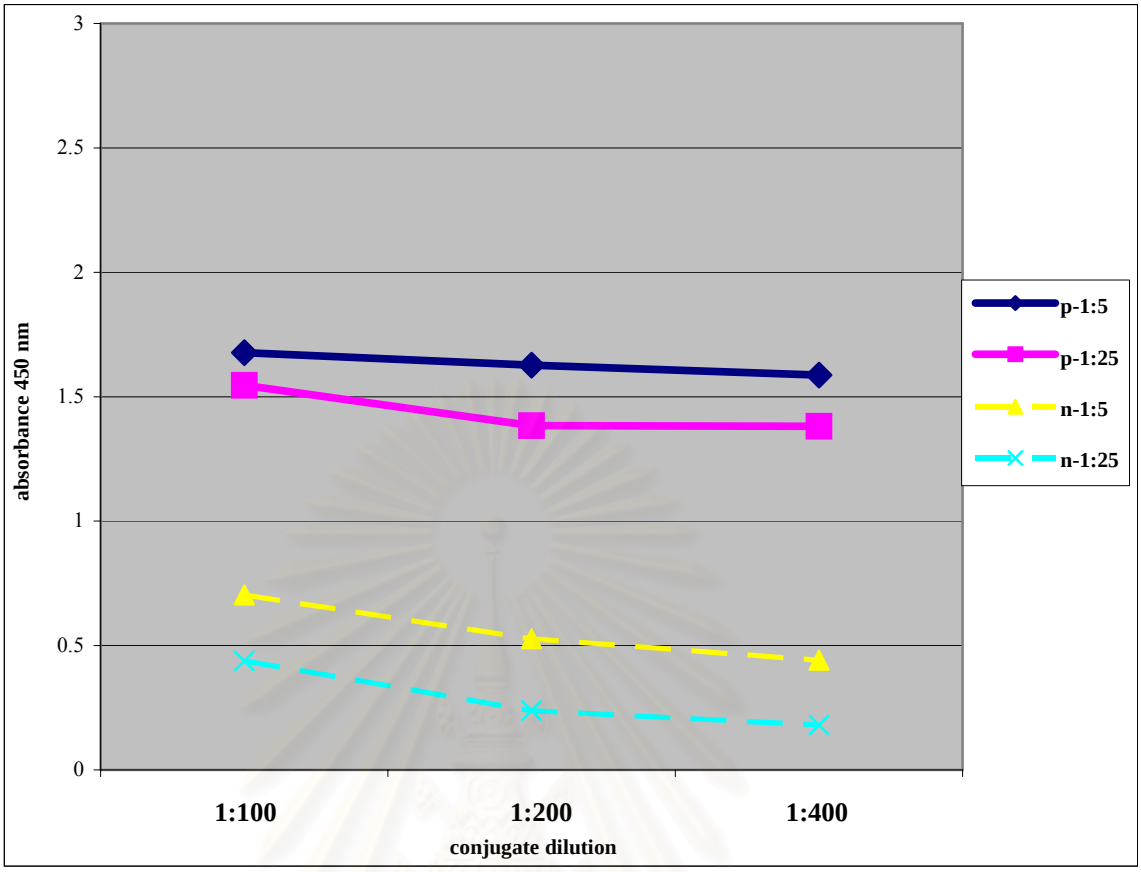


Fig 9. Checkerboard titration for IgE antibody against advanced L3s *G.spinigerum* antigen. Absorbance at 450 nm obtained by ELISA assays using pooled infected sera (P) and negative control sera (n) is shown using straight lines and dot lines, respectively. Plates were coated with 50 µl of antigen at concentration 5 µg/ml. Dilutions of sera used were 1:5 and 1:25. The dilutions of conjugate rat anti-mouse immunoglobulin E were 1:100, 1:200 and 1:400 and HRP-rabbit anti-rat immunoglobulin G was 1:2,000.

Table 1. Optimal conditions of ELISA test for the detection of IgG, IgG subclass 1, IgG subclass 2a and IgE antibody

Step of ELISA test	IgG		IgG1		IgG2a		IgE	
	Concentration or dilution	Reaction time	Concentration or dilution	Reaction time	Concentration or dilution	Reaction time	Concentration or dilution	Reaction time
Coating of L3s antigen	5 μg/ml	18 hr	5 μg/ml	18 hr	5 μg/ml	18 hr	5 μg/ml	18 hr
Reacting with tested sera	1:125	1 hr	1:125	1 hr	1:125	1 hr	1:25	2 hr
Reacting with conjugate 1	1:2,000	1 hr	1:2,000	1 hr	1:1,000	1 hr	1:400	1 hr
Reacting with conjugate 2	–	–	–	–	–	–	1:2,000	1 hr
Color development	TMB	10 min	TMB	10 min	TMB	15 min	TMB	10 min

1.2 ELISA antibody level of mice infected with *G. spinigerum*

In this experiment, mice were infected with two doses of 10 and 20 larvae. To detect the antibody level of IgG, IgG subclass 1 and subclass 2a and IgE specific to advanced L3s antigen. ELISA assays were performed using sera from infected mice with or without ivermectin treatment. Ten day after infection with 10 or 20 larvae *G.spinigerum*, mice were divided into two groups. The first group comprising half of the mice was treated with 0.4 mg/kg body weight ivermectin orally. Blood was taken from both groups at different time points of Day 10, 30, 60, 90, 120, 150 and 180 post infection (PI) and post drug treatment (PRx).

1.2.1 Kinetic of IgG antibody response

The results presented in Fig 10 and Fig 12 showed that the IgG ELISA value of infected mice sera was statistically significant higher than those of healthy controls. From all of the infected sera, the antibody could be detected within Day 10 PI. Thereafter it was increased gradually and the mean value of the infected group reached a plateau on Day 150 and 120 PI of 10 and 20 larvae infected mice (OD = 1.804 and OD = 2.089 respectively, Table 2). From the peak onward the response gradually decreased through to the end of the experiment. The mean IgG-Ab level of all the 20 larvae infected mice was statistically significant higher than those of 10 larvae infected group at various time points [Day 10 (P=0.001), Day 60 (P=0.015), Day 120 (P=0.047) and 180 PI (P=0.031), Fig 12].

With regard to the presence of IgG-Ab in 10 larvae after ivermectin treatment (Fig 11 and 13), the mean levels could continually increase and was statistically significant higher than the corresponding pre-treatment value of Day 10 and Day 30 post drug treatment (PRx, P=0.006 and P=0.044, respectively, Fig 13). At Day 60 PRx, the level was still high but thereafter it was gradually decreased. However, it was statistically significant higher than the normal animal in every time points of post drug treatment sera. For the 20 larvae infected animals, after being treated with ivermectin, the mean IgG ELISA value could also continually increase, at Day 60 PRx onward, the level showed statistically significant lower than the correspond pretreatment value [Day 60 (P=0.010), Day 90 (P=0.006), Day 120 (P=0.047) and Day 180 (P=0.004), Fig 14].

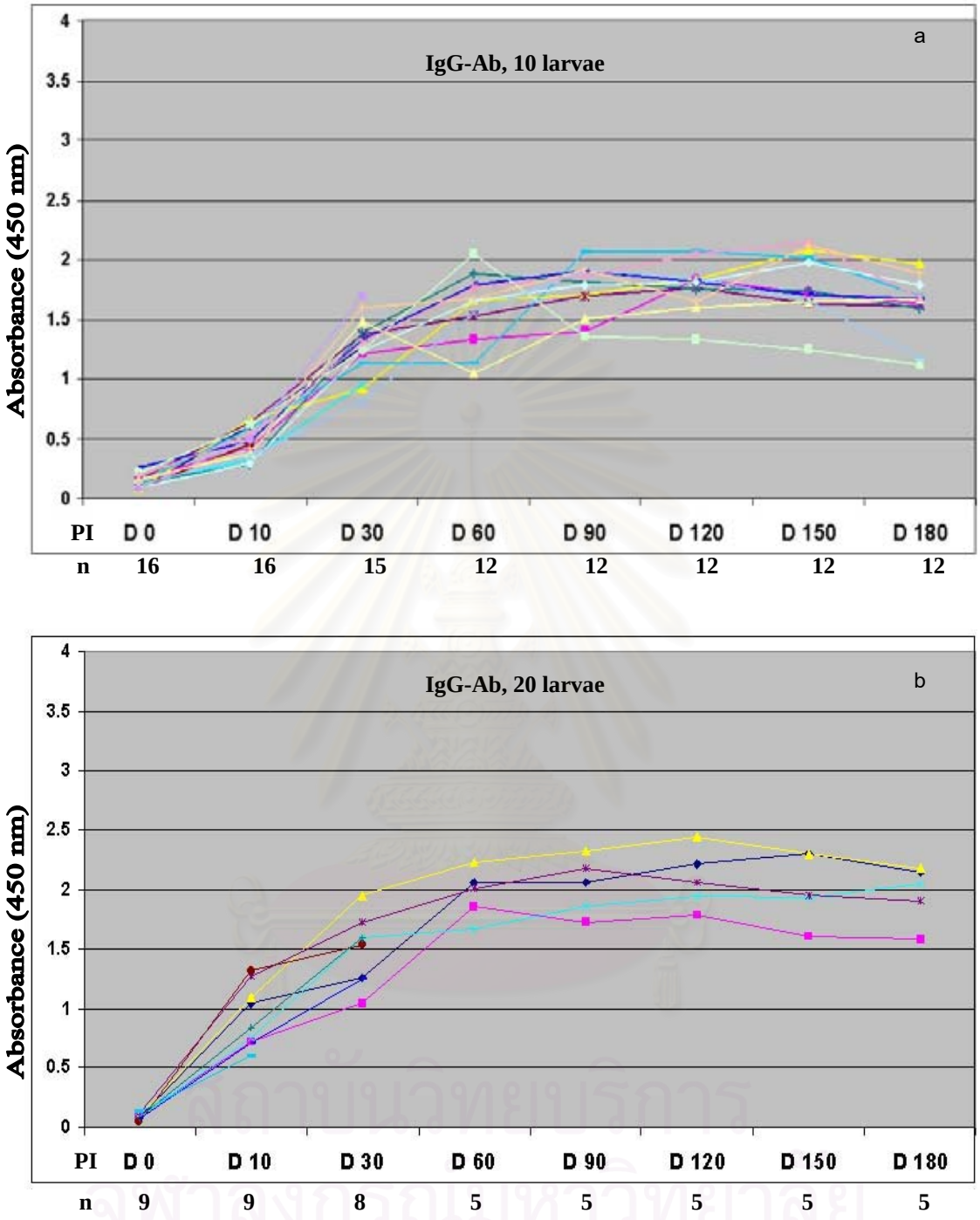


Fig 10. Kinetic of optical density of ELISA for IgG antibody for somatic L3s antigen in mice infected with 10 (a) and 20 (b) larvae of *G.spinigerum* at different time points (D 10, 30, 60, 90, 120, 150 and 180 PI). Each line represents OD values of individual animal; n is the number of animal.

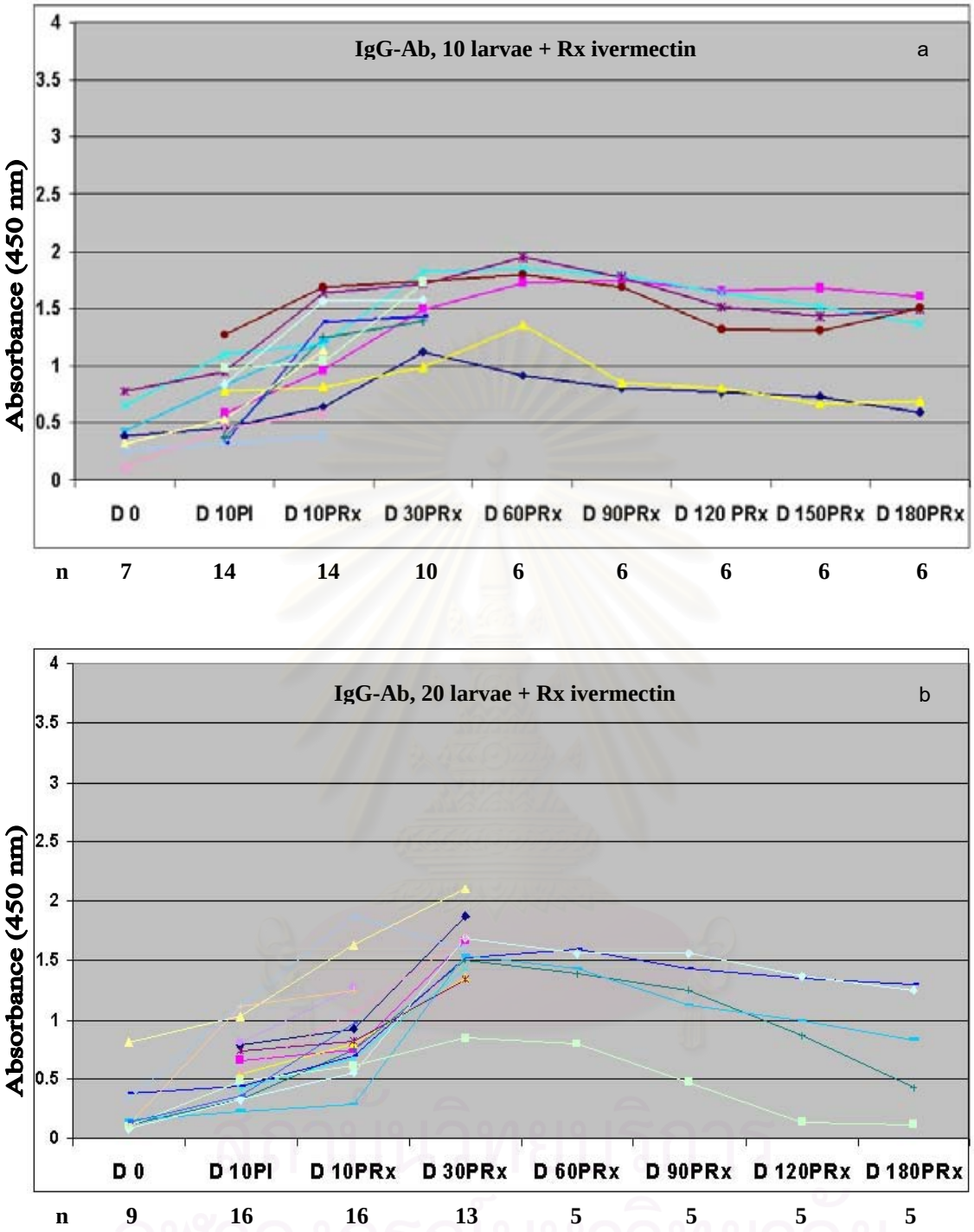


Fig 11. Kinetic of optical density of ELISA for IgG antibody for somatic L3s antigen in treated infected mice with 10 (a) and 20 (b) larvae of *G.spinigerum* at different time points (D 10, 30, 60, 90, 120, 150 and 180 PRx). Each line represents OD values of individual animal; n is the number of animal.

Table 2. Absorbance values of specific IgG antibody in serum of mice infected with *G.spinigerum* at different time intervals.

Group of mice		Absorbance density (450 nm)								
		D 0	D 10 PI	D 10 PRx	D 30 PI, PRx	D 60 PI, PRx	D 90 PI, PRx	D 120 PI, PRx	D 150 PI, PRx	D 180 PI, PRx
10 larvae	N	16	16		15	12	12	12	12	12
	X	0.137	0.470	-	1.263	1.590	1.741	1.779	1.804	1.623
	SD	0.050	0.124		0.242	0.294	0.219	0.196	0.263	0.248
20 larvae	N	9	9		8	5	5	5	5	5
	X	0.091	0.925	-	1.492	1.962	2.027	2.089	2.020	1.969
	SD	0.026	0.261		0.295	0.215	0.244	0.250	0.292	0.245
10 larvae	N	7	14	14	10	6	6	6	6	6
+ Rx	X	0.420	0.697	1.103	1.496	1.594	1.440	1.279	1.221	1.206
ivermectin	SD	0.227	0.306	0.399	0.276	0.392	0.481	0.407	0.421	0.445
20 larvae	N	9	16	16	13	5	5	5		5
+ Rx	X	0.245	0.623	0.932	1.550	1.352	1.167	0.939	-	0.784
ivermectin	SD	0.237	0.287	0.407	0.298	0.322	0.420	0.501		0.517

N = Number tested

X = Mean of Absorbance density

SD = Standard deviation of the mean

PI = Post infection

PRx = Post treatment

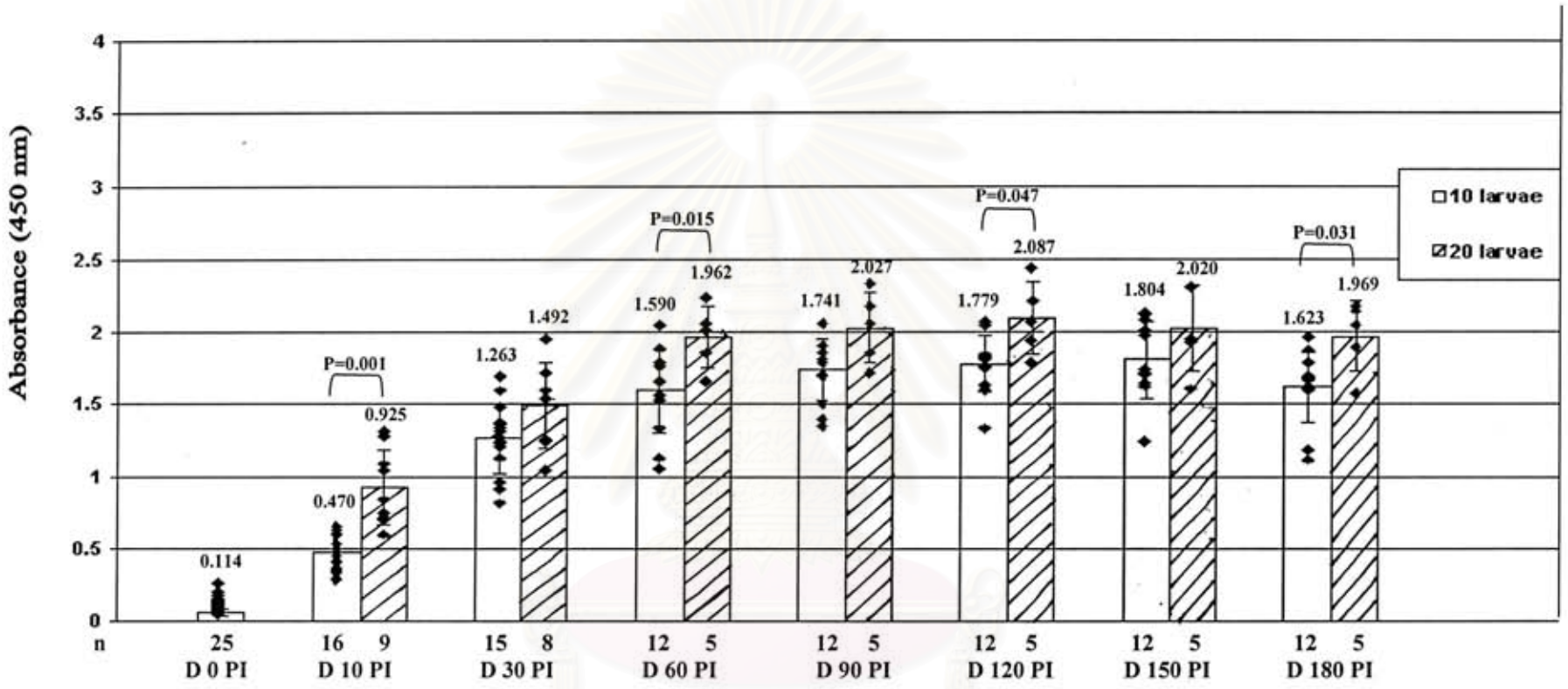


Fig 12. Optical density value of ELISA for IgG antibody to somatic L3s antigen in mice infected with 10 and 20 larvae of *G.spinigerum* at different time points (D 10, 30, 60, 90, 120, 150 and 180 PI). Bars and lines represent mean and SD of individual animal indicated (•); n is the number of animal.

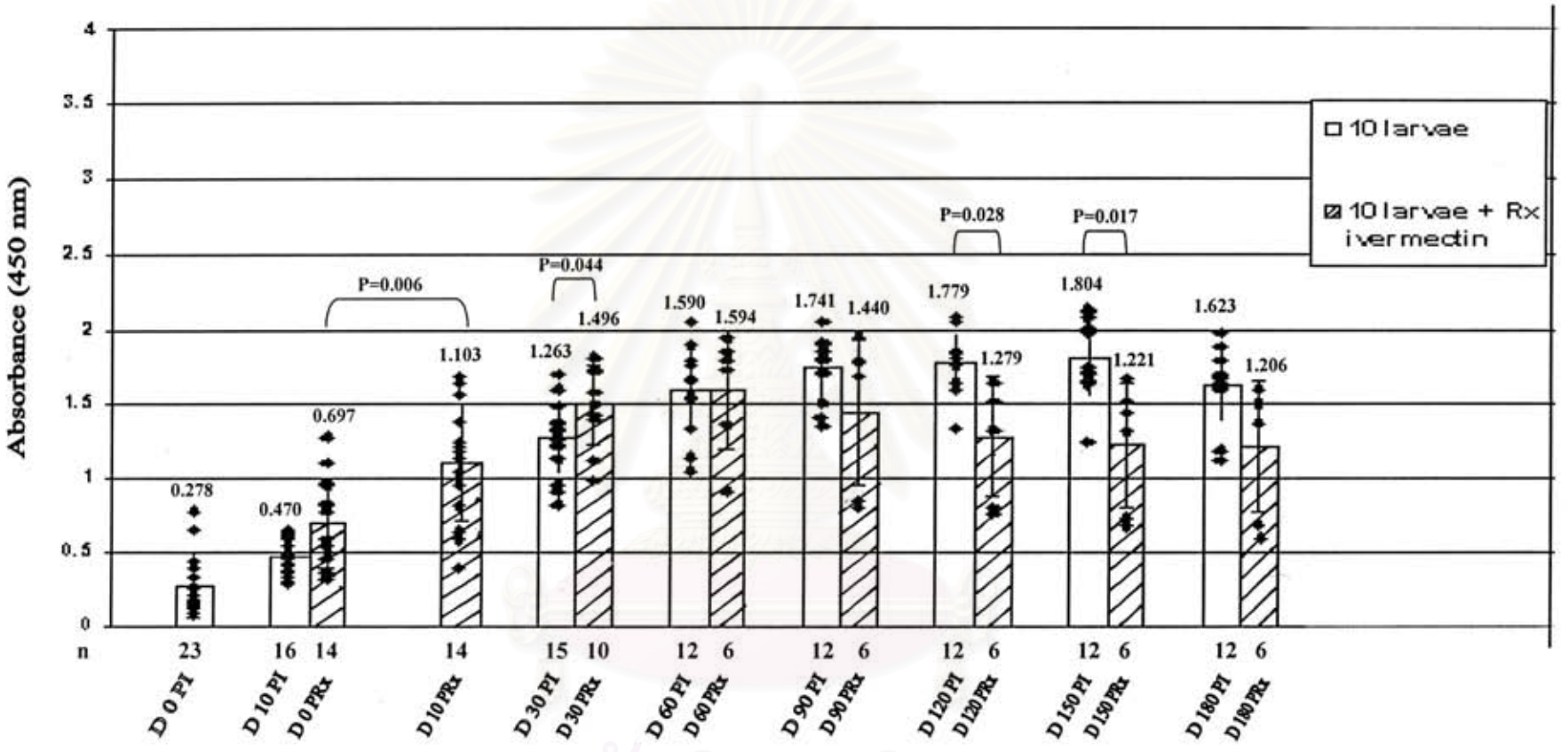


Fig 13. Optical density value of ELISA for IgG antibody to somatic L3s antigen in mice infected with 10 larvae and treated infected mice with 10 larvae of *G.spinigerum* at different time points (D 10, 30, 60, 90, 120, 150 and 180 PI, PRx). Bars and lines represent mean and SD of individual animal indicated (•); n is the number of animal. PI : post infection; PRx : post drug treatment.

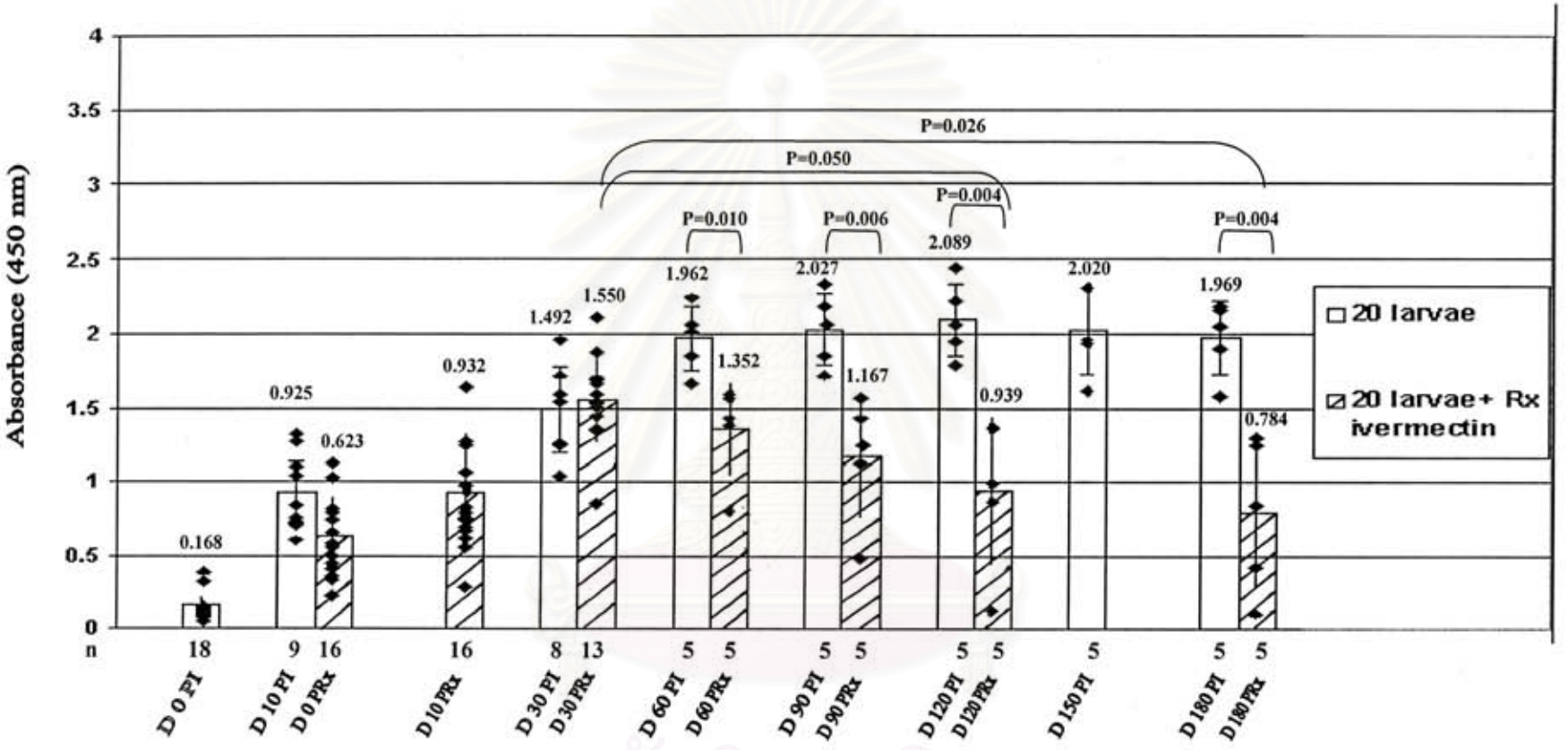


Fig 14. Optical density value of ELISA for IgG antibody to somatic L3s antigen in mice infected with 20 larvae and treated infected mice with 20 larvae of *G.spinigerum* at different time points (D 10, 30, 60, 90, 120, 150 and 180 PI, PRx). Bars and lines represent mean and SD of individual animal indicated (•); n is the number of animal. PI : post infection; PRx : post drug treatment.

1.2.2 Kinetic of IgG subclass 1 antibody response

The Kinetic of IgG1-Ab was considerably similar to that of IgG-Ab (Fig 15 and Fig 16). The level of IgG1 of the infected animal could be detected within Day 10 PI, slightly increased and reached a plateau on Day 90 PI and Day 60 PI of 10 larvae (OD = 2.351) and 20 larvae infected groups (OD = 2.354) respectively (Table 3). Thereafter, the level decreased gradually and statistically significant decreased from the peak at Day 180 PI (P=0.034, Fig 17). The mean ELISA value of the 10 larvae infected group was no statistically significant lower than those of the 20 larvae infected group (Fig 17).

After the 10 larvae infected mice being treated with anthelmintic drug at Day 10 PI, the IgG1-Ab levels remained increase until Day 30 PRx and then gradually decrease thereafter through to the end of the experiment. The level showed statistically significant decrease starting from Day 90 [Day 90 (P=0.008), Day 120 (P=0.001), Day 150 (P=0.001) and Day 180 (P=0.009), Fig 18]. For the 20 larvae infected animals, after being treated with ivermectin, the mean IgG1 ELISA value could also continually increase. Thereafter, the level decreased gradually and statistically significant decreased from the peak at Day 180 PRx (P=0.015, Fig 19).

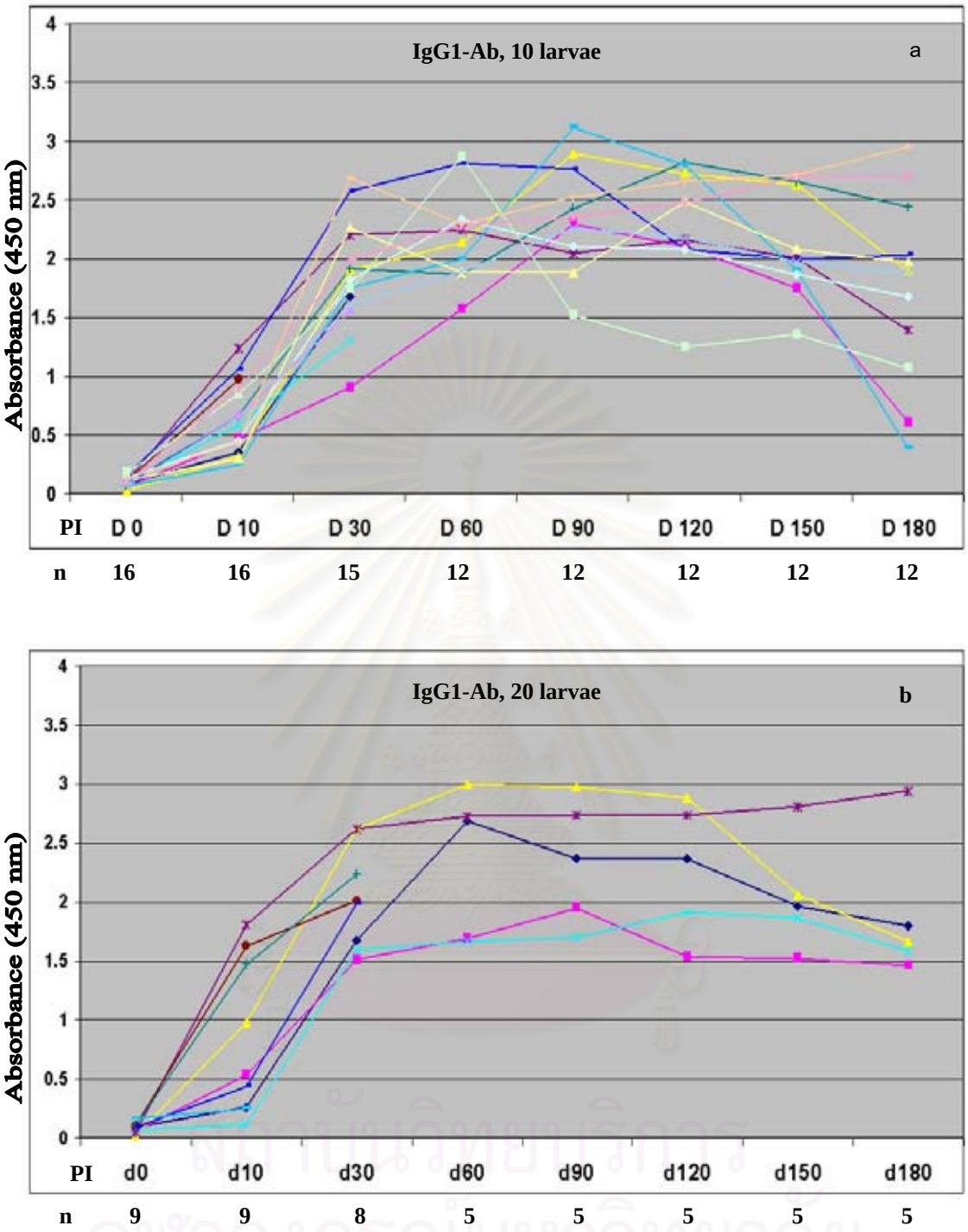


Fig 15. Kinetic of optical density of ELISA for IgG subclass 1 antibody to somatic L3s antigen in mice infected with 10 (a) and 20 (b) larvae of *G.spinigerum* at different time points (D 10, 30, 60, 90, 120, 150 and 180 PI). Each line represents OD values of individual animal; n is the number of animal.

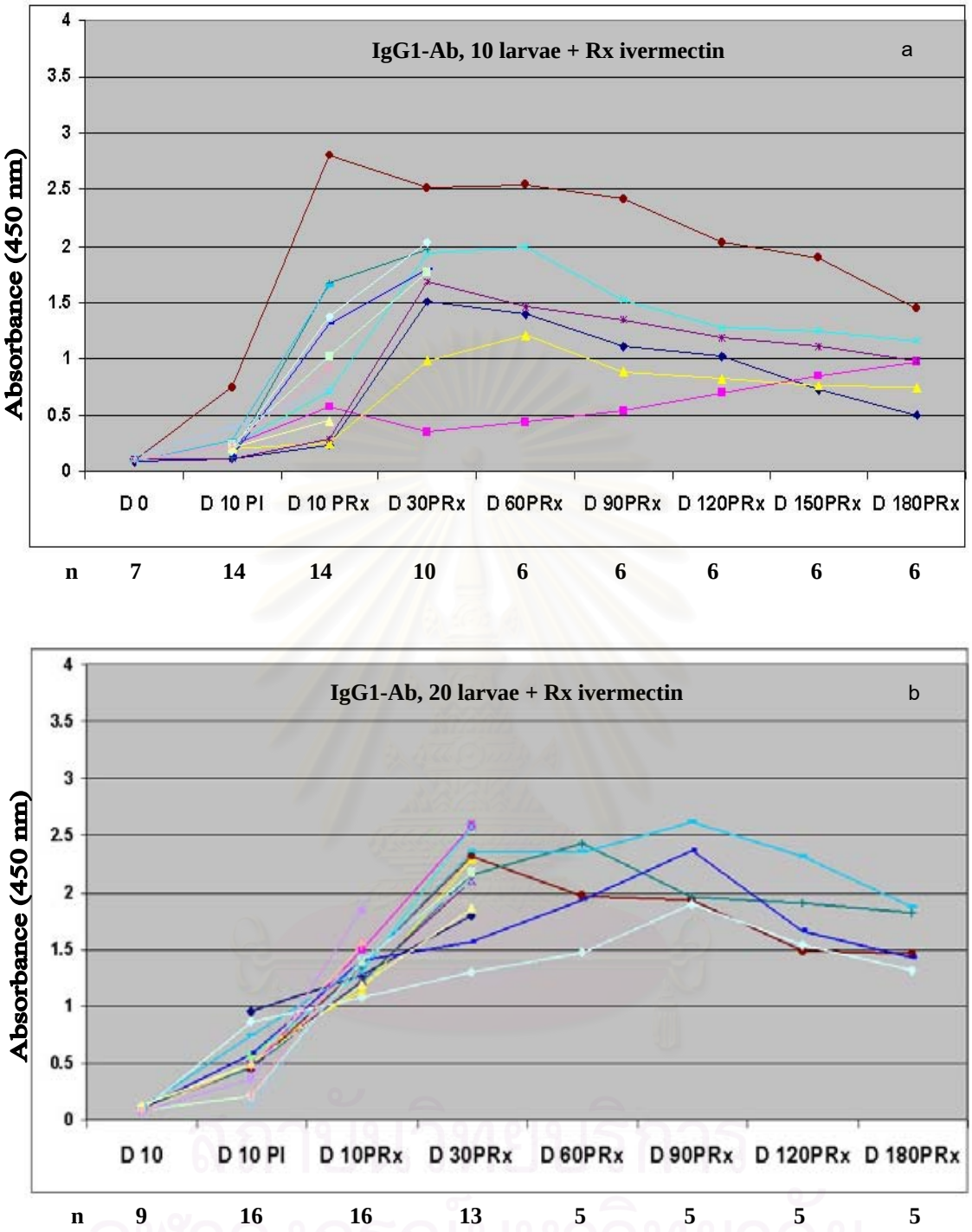


Fig 16. Kinetic of optical density of ELISA for IgG subclass 1 antibody to somatic L3s antigen in treated infected mice with 10 (a) and 20 (b) larvae of *G.spinigerum* at different time points (D 10, 30, 60, 90, 120, 150 and 180 PRx). Each line represents OD values of individual animal; n is the number of animal.

Table 3 Absorbance values of specific IgG subclass 1 antibody in serum of mice infected with *G.spinigerum* at different time intervals.

Group of mice		Absorbance density (450 nm)								
		D 0	D 10 PI	D 10 PRx	D 30 PI, PRx	D 60 PI, PRx	D 90 PI, PRx	D 120 PI, PRx	D 150 PI, PRx	D 180 PI, PRx
10 larvae	N	16	16	-	15	12	12	12	12	12
	X	0.102	0.636		1.858	2.183	2.351	2.318	2.138	1.753
	SD	0.040	0.297		0.458	0.380	0.443	0.444	0.436	0.783
20 larvae	N	9	9	-	8	5	5	5	5	5
	X	0.086	0.833		2.035	2.354	2.346	2.289	2.046	1.891
	SD	0.041	0.654		0.435	0.630	0.527	0.561	0.474	0.601
10 larvae + Rx ivermectin	N	7	14	14	10	6	6	6	6	6
	X	0.103	0.246	0.100	1.654	1.506	1.303	1.172	1.095	0.967
	SD	0.017	0.162	0.716	0.604	0.717	0.648	0.473	0.443	0.329
20 larvae + Rx ivermectin	N	9	16	16	13	5	5	5		5
	X	0.098	0.504	1.362	2.089	2.033	2.155	1.783	-	1.580
	SD	0.016	0.222	0.191	0.374	0.386	0.322	0.341		0.253

N = Number tested

X = Mean of Absorbance density

SD = Standard deviation of the mean

PI = Post infection

PRx = Post drug treatment

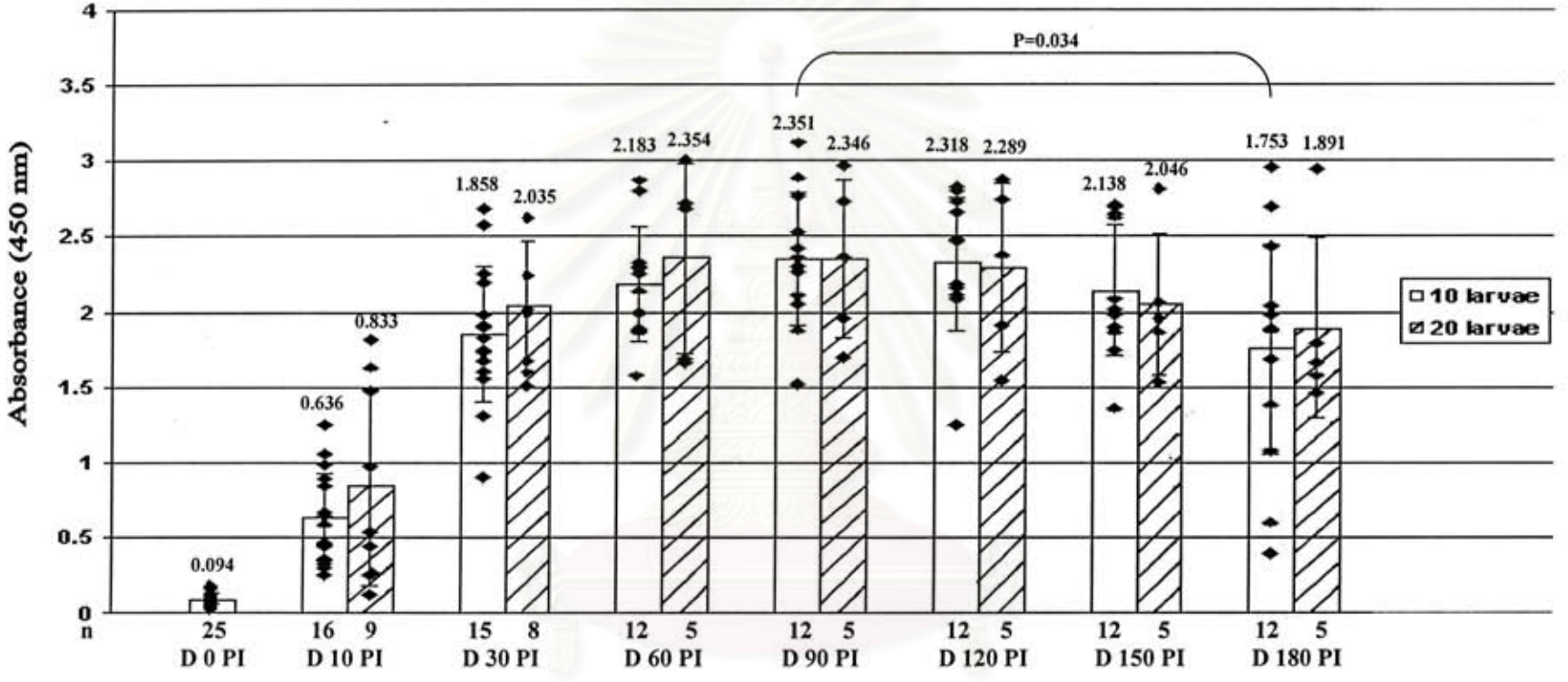


Fig 17. Optical density value of ELISA for IgG subclass 1 antibody to somatic L3s antigen in mice infected with 10 and 20 larvae of *G.spinigerum* at different time points (D 10, 30, 60, 90, 120, 150 and 180 PI). Bars and lines represent mean and SD of individual animal indicated (•); n is the number of animal.

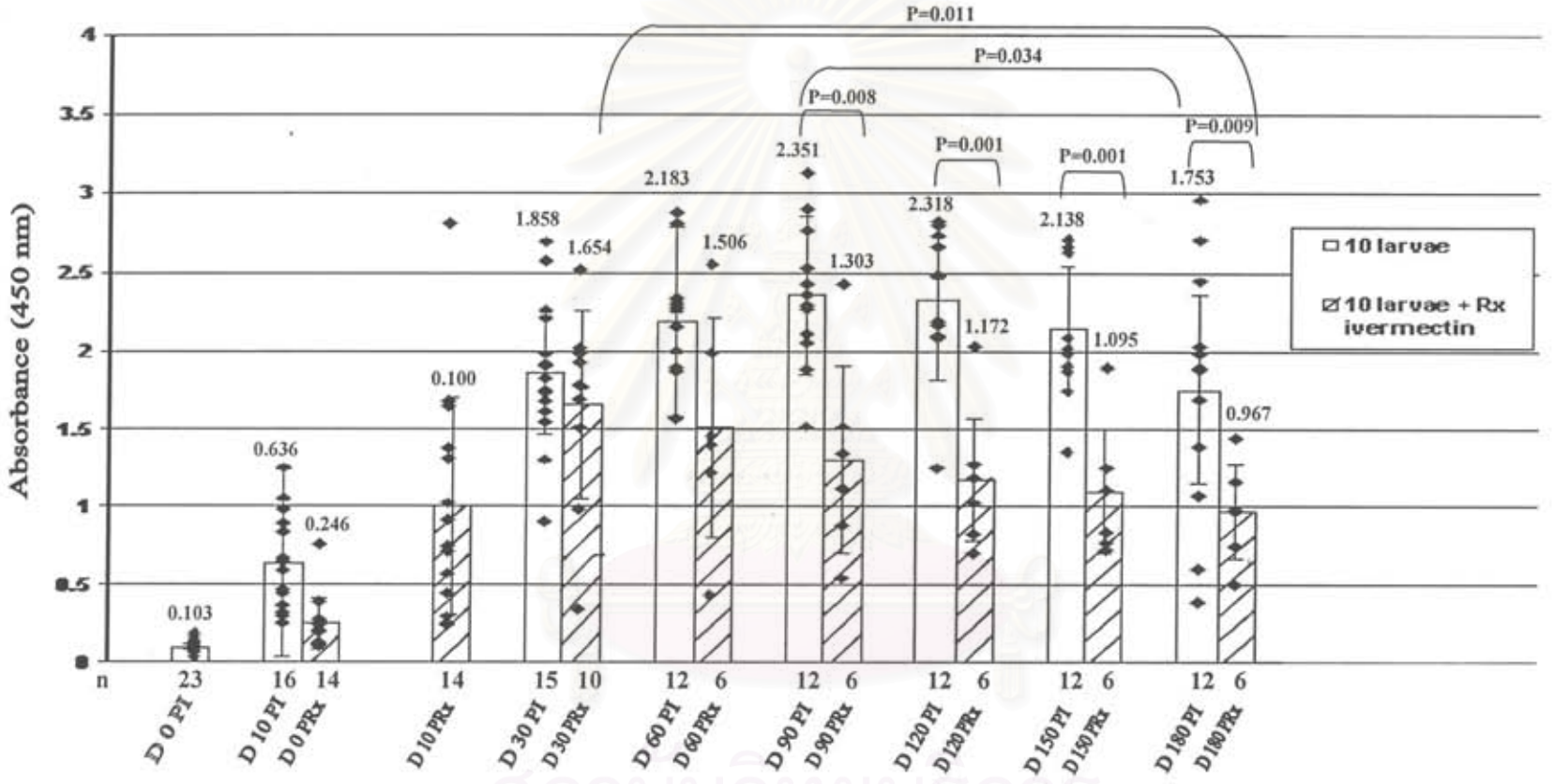


Fig 18. Optical density value of ELISA for IgG subclass 1 antibody to somatic L3s antigen in mice infected with 10 larvae and treated infected 10 larvae of *G.spinigerum* at different time points (D 10, 30, 60, 90, 120, 150 and 180 PI, PRx). Bars and lines represent mean and SD of individual animal indicated (•); n is the number of animal. PI: post infection; PRx: post drug treatment.

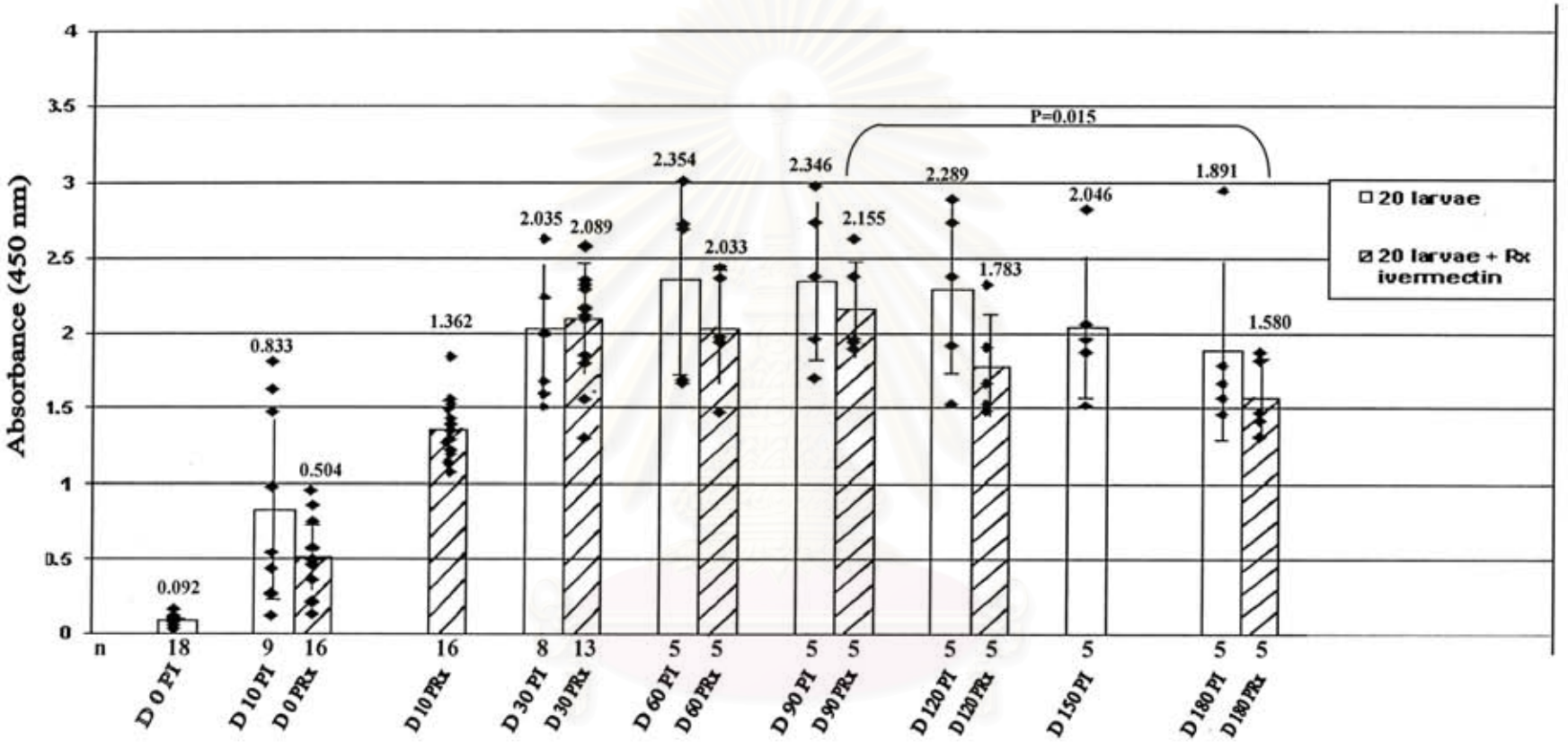


Fig 19. Optical density value of ELISA for IgG subclass 1 antibody to somatic L3s antigen in mice infected with 20 larvae and treated infected 20 larvae of *G.spinigerum* at different time points (D 10, 30, 60, 90, 120, 150 and 180 PI, PRx). Bars and lines represent mean and SD of individual animal indicated (•); n is the number of animal. PI: post infection; PRx: post drug treatment.

1.2.3 Kinetic of IgG subclass 2a antibody response

The mean ELISA value of IgG2a from all the infected mice sera could be detected within Day 10 PI, slightly increased thereafter and could reach a short peak on day 120 PI (Table 4, Fig 20 and Fig 23).

After the 10 larvae infected group being treated with ivermectin, the mean value of IgG2a still had no statistically significant change form the corresponding pretreatment value. This same pattern was also observed in the group of 20 larvae infected and drug treated (Fig 22, Fig 23 and Fig 24).

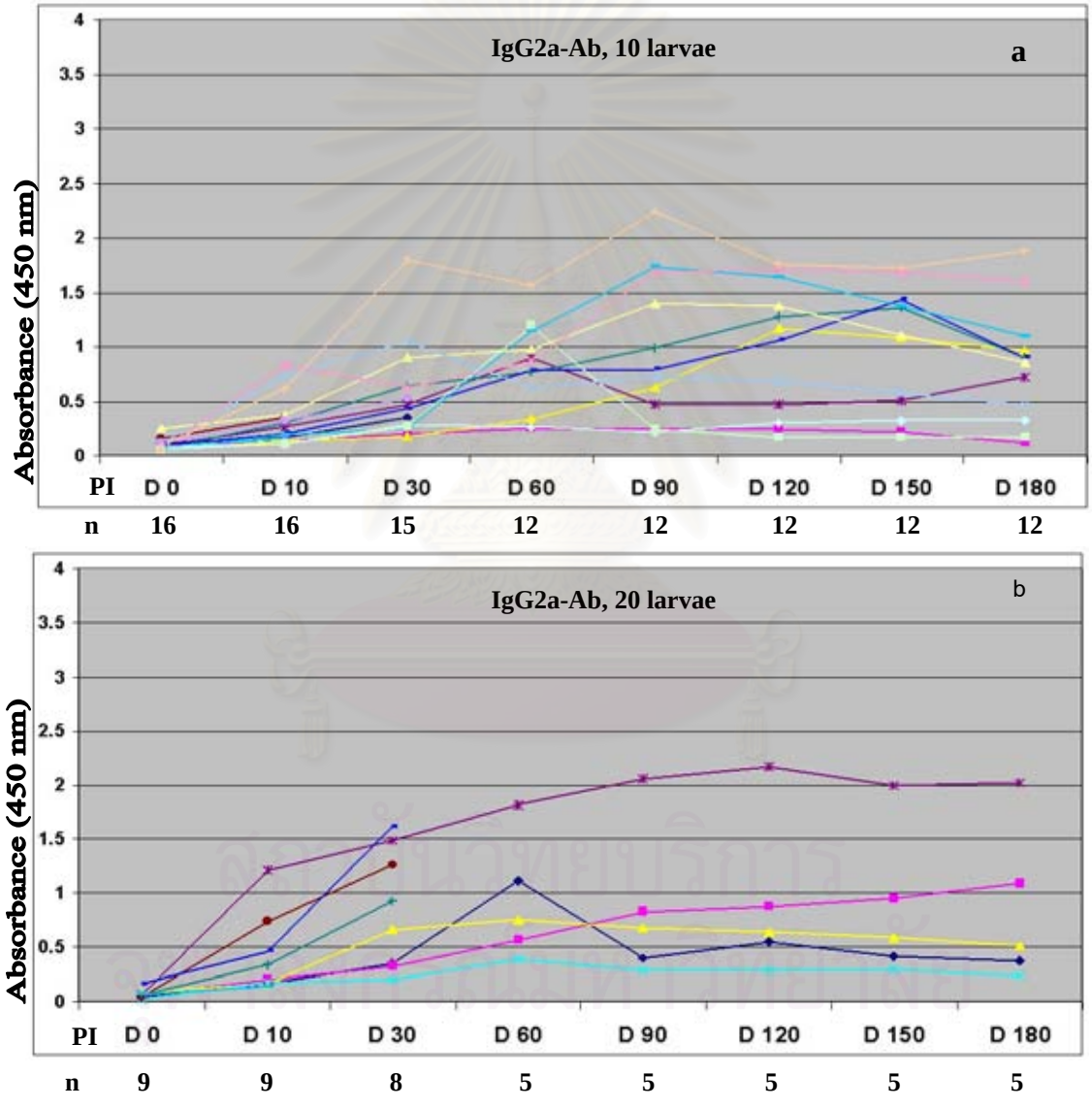


Fig 20. Kinetic of optical density of ELISA for IgG subclass 2a antibody to somatic L3s antigen in mice infected with 10 (a) and 20 (b) larvae of *G.spinigerum* at different time points (D 10, 30, 60, 90, 120, 150 and 180 PI). Each line represents OD values of individual animal; n is the number of animal.

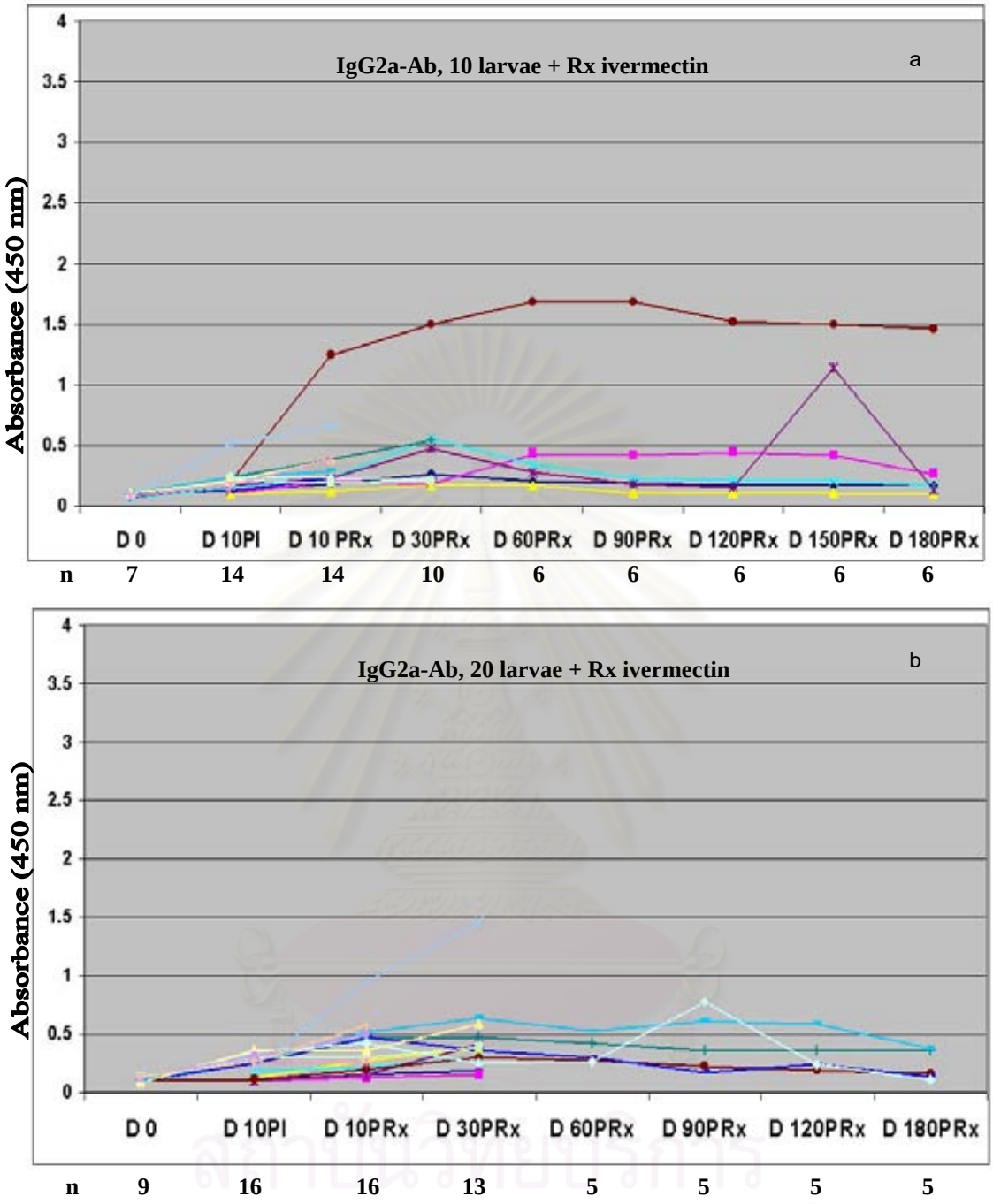


Fig 21. Kinetic of optical density of ELISA for IgG subclass 2a antibody to somatic L3s antigen in treated infected mice with 10 (a) and 20 (b) larvae of *G.spinigerum* at different time points (D 10, 30, 60, 90, 120, 150 and 180 PRx). Each line represents OD values of individual animal; n is the number of animal.

Table 4 Absorbance values of specific IgG subclass 2a antibody in serum of mice infected with *G.spinigerum* at different time intervals.

Group of mice		Absorbance density (450 nm)								
		D 0	D 10 PI	D 10 PRx	D 30 PI, PRx	D 60 PI, PRx	D 90 PI, PRx	D 120 PI, PRx	D 150 PI, PRx	D 180 PI, PRx
10 larvae	N	16	16		15	12	12	12	12	12
	X	0.100	0.314	-	0.572	0.807	0.945	0.988	0.964	0.834
	SD	0.049	0.225		0.436	0.397	0.673	0.592	0.577	0.533
20 larvae	N	9	9		8	5	5	5	5	5
	X	0.073	0.398	-	0.856	0.926	0.850	0.905	0.854	0.848
	SD	0.038	0.366		0.557	0.564	0.700	0.740	0.686	0.732
10 larvae	N	7	14	14	10	6	6	6	6	6
+ Rx	X	0.083	0.197	0.350	0.431	0.515	0.465	0.432	0.588	0.380
ivermectin	SD	0.019	0.102	0.292	0.405	0.579	0.607	0.545	0.488	0.532
20 larvae	N	9	16	16	13	5	5	5		5
+ Rx	X	0.108	0.223	0.372	0.452	0.356	0.429	0.321	-	0.230
ivermectin	SD	0.017	0.089	0.213	0.334	0.117	0.253	0.161		0.130

N = Number tested

X = Mean of Absorbance density

SD = Standard deviation of the mean

PI = Post infection

PRx = Post drug treatment

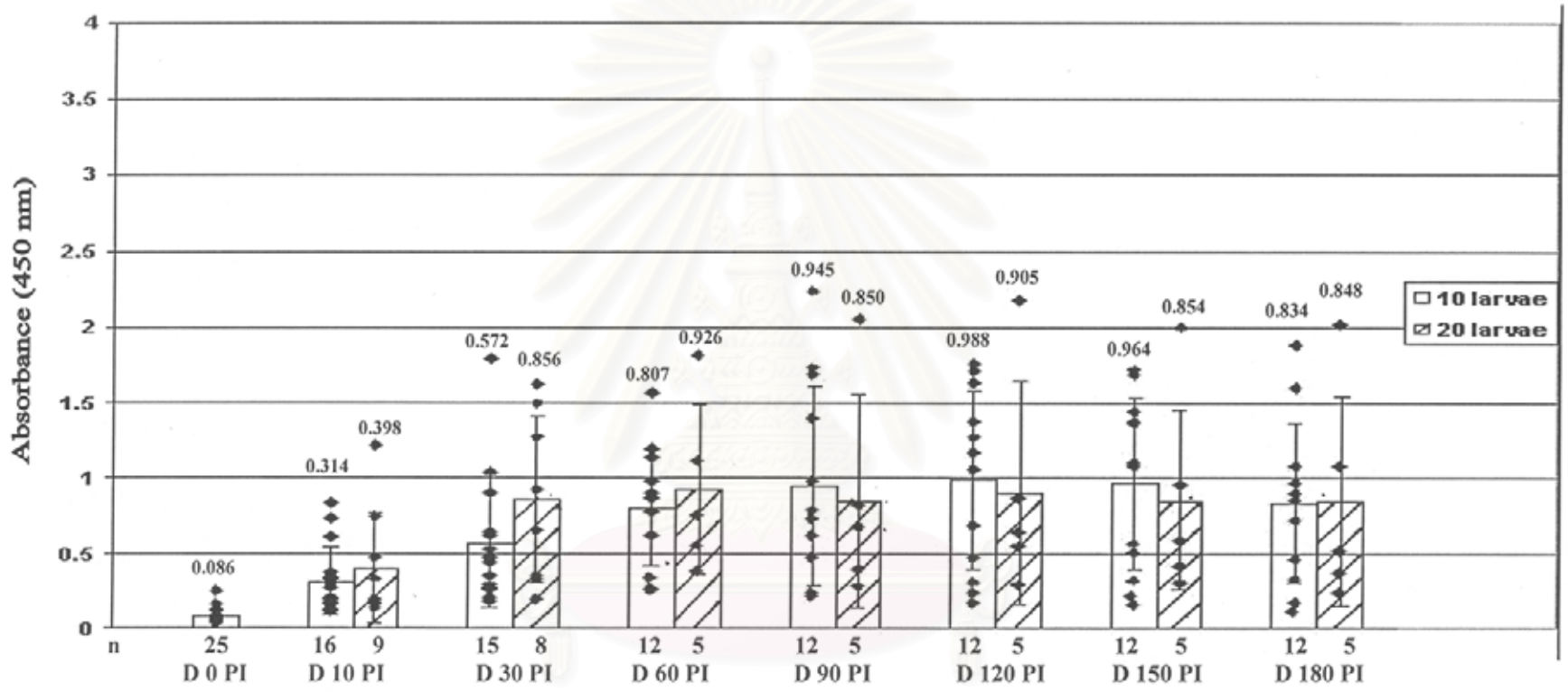


Fig 22. Optical density value of ELISA for IgG subclass 2a antibody to somatic L3s antigen in mice infected with 10 and 20 larvae of *G.spinigerum* at different time points (D 10, 30, 60, 90, 120, 150 and 180 PI). Bars and lines represent mean and SD of individual animal indicated (•); n is the number of animal.

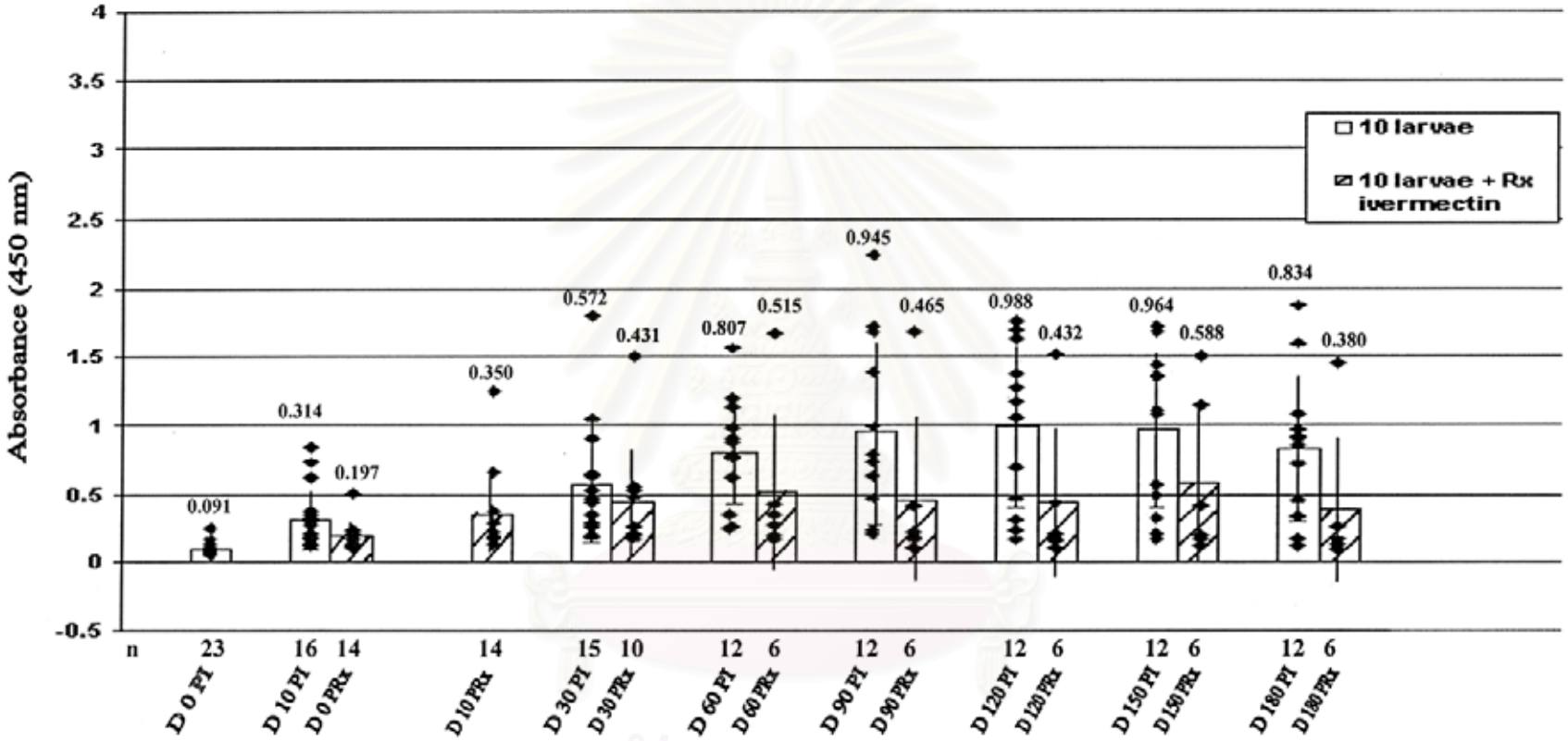


Fig 23. Optical density value of ELISA for IgG subclass 2a antibody to somatic L3s antigen in mice infected with 10 larvae and treated infected 10 larvae of *G.spinigerum* at different time points (D 10, 30, 60, 90, 120, 150 and 180 PI, PRx). Bars and lines represent mean and SD of individual animal indicated (•); n is the number of animal. PI: post infection; PRx: post drug treatment.

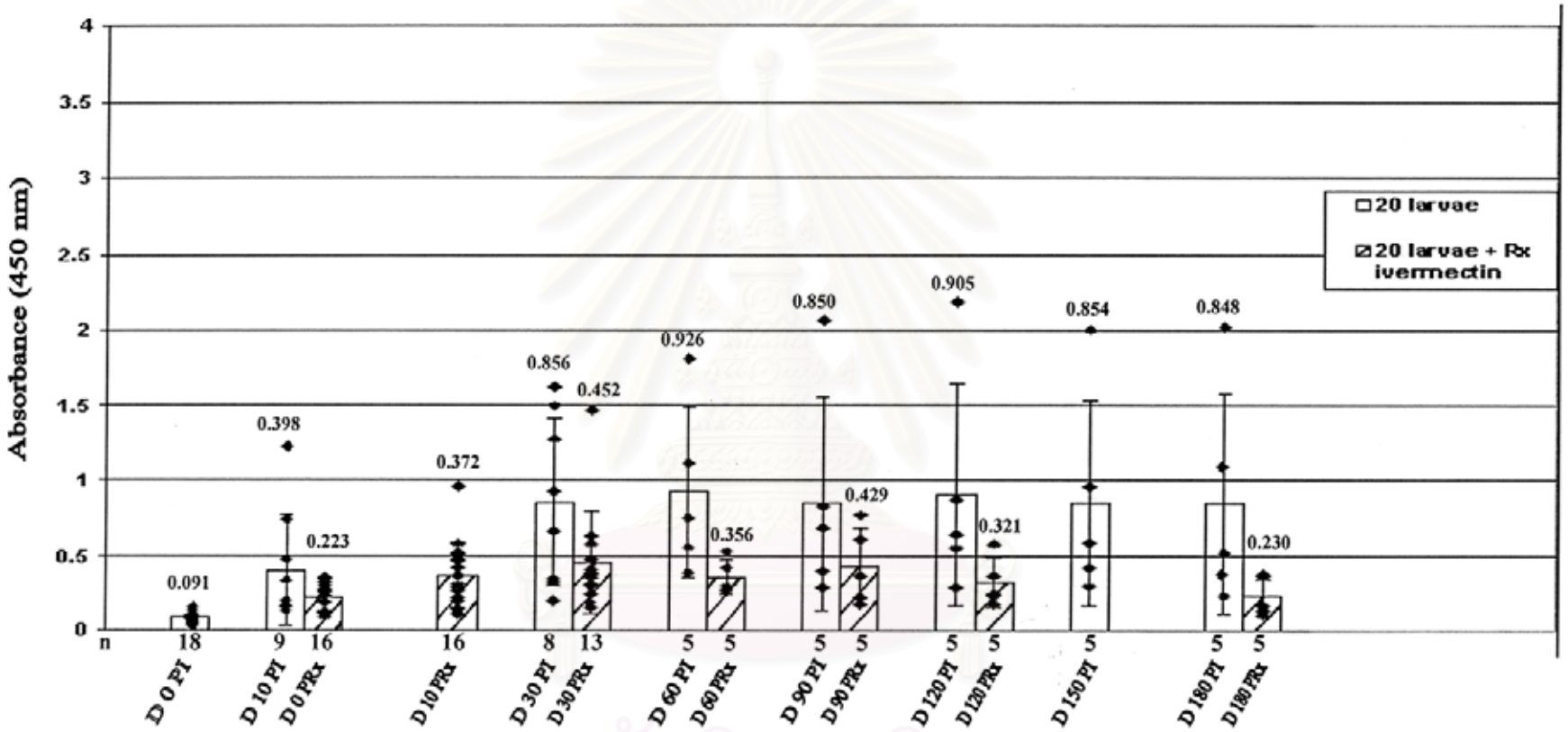


Fig 24. Optical density value of ELISA for IgG subclass 2a antibody to somatic L3s antigen in mice infected with 20 larvae and treated infected 20 larvae of *G.spinigerum* at different time points (D 10, 30, 60, 90, 120, 150 and 180 PI, PRx). Bars and lines represent mean and SD of individual animal indicated (•); n is the number of animal. PI: post infection; PRx: post drug treatment.

1.2.4 Kinetic of IgE antibody response

The IgE antibody level from all of the infected mice could be detected within Day 10 PI (Fig 25). Thereafter, the level increased gradually and reached a plateau on Day 90 PI of both the 10 larvae and 20 larvae infected groups (OD = 1.486 and OD = 1.340 respectively, Table 5). Later on, the level continually decreased but had no statistically significant difference neither from the highest value nor from the value of any time points (Fig 27).

After the 10 larvae infected group being treated with ivermectin, the pattern was similar to that of IgG1. Since the mean value of IgE could continually increased from Day 10 PRx until Day 60 PRx. Thereafter, it had lower than those of the corresponding pretreatment value (Fig 28). In addition, the mean IgE value of the 20 larvae infected group could demonstrate the drastically statistically significant decrease at Day 90 PRx and onward [Day 90 (P=0.006), Day 120 (P=0.009) and Day 180 (P=0.038), Fig 29].

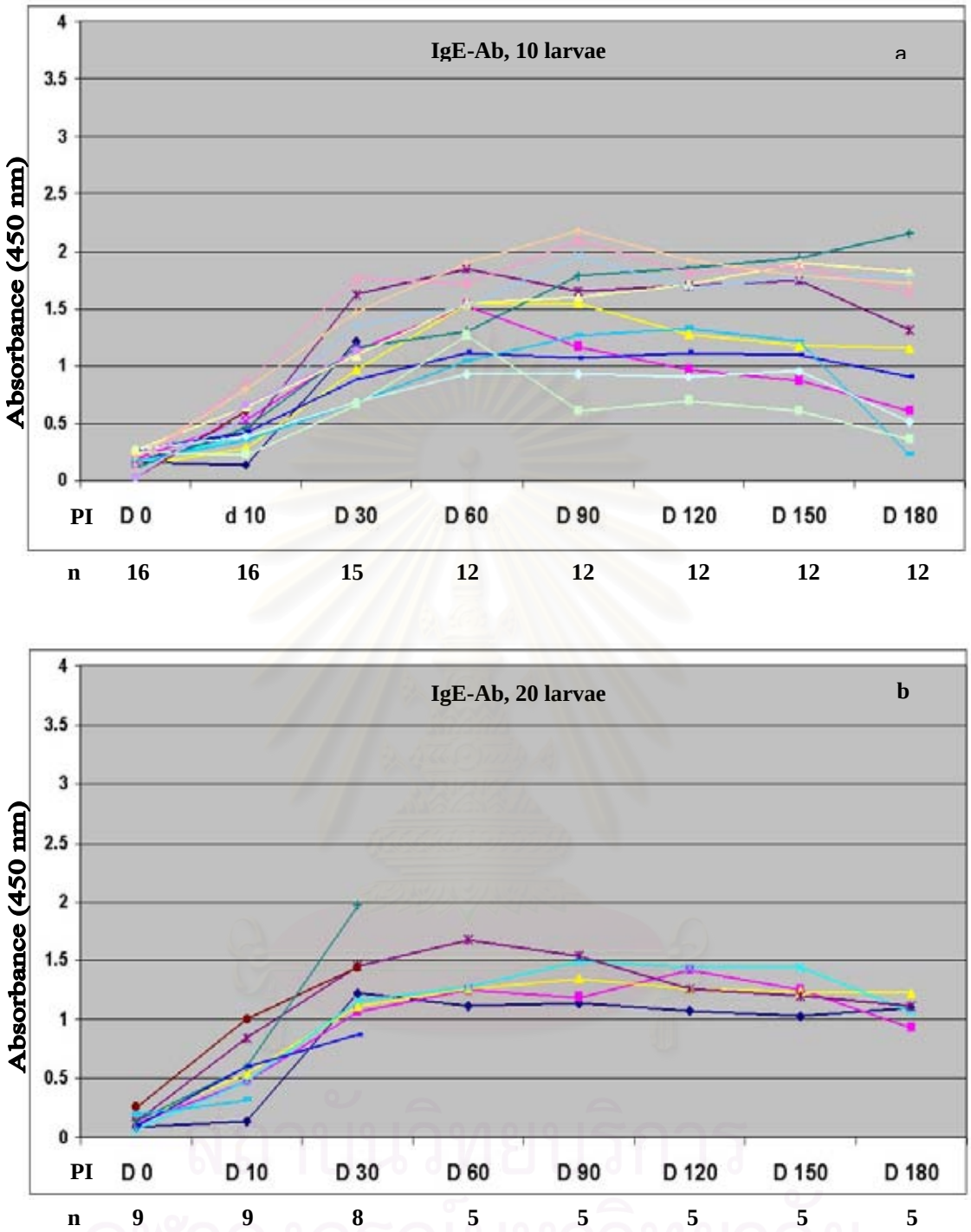


Fig 25. Kinetic of optical density of ELISA for IgE antibody to somatic L3s antigen in mice infected with 10 (a) and 20 (b) larvae of *G.spinigerum* at different time points (D 10, 30, 60, 90, 120, 150 and 180 PI). Each line represents OD values of individual animal; n is the number of animal.

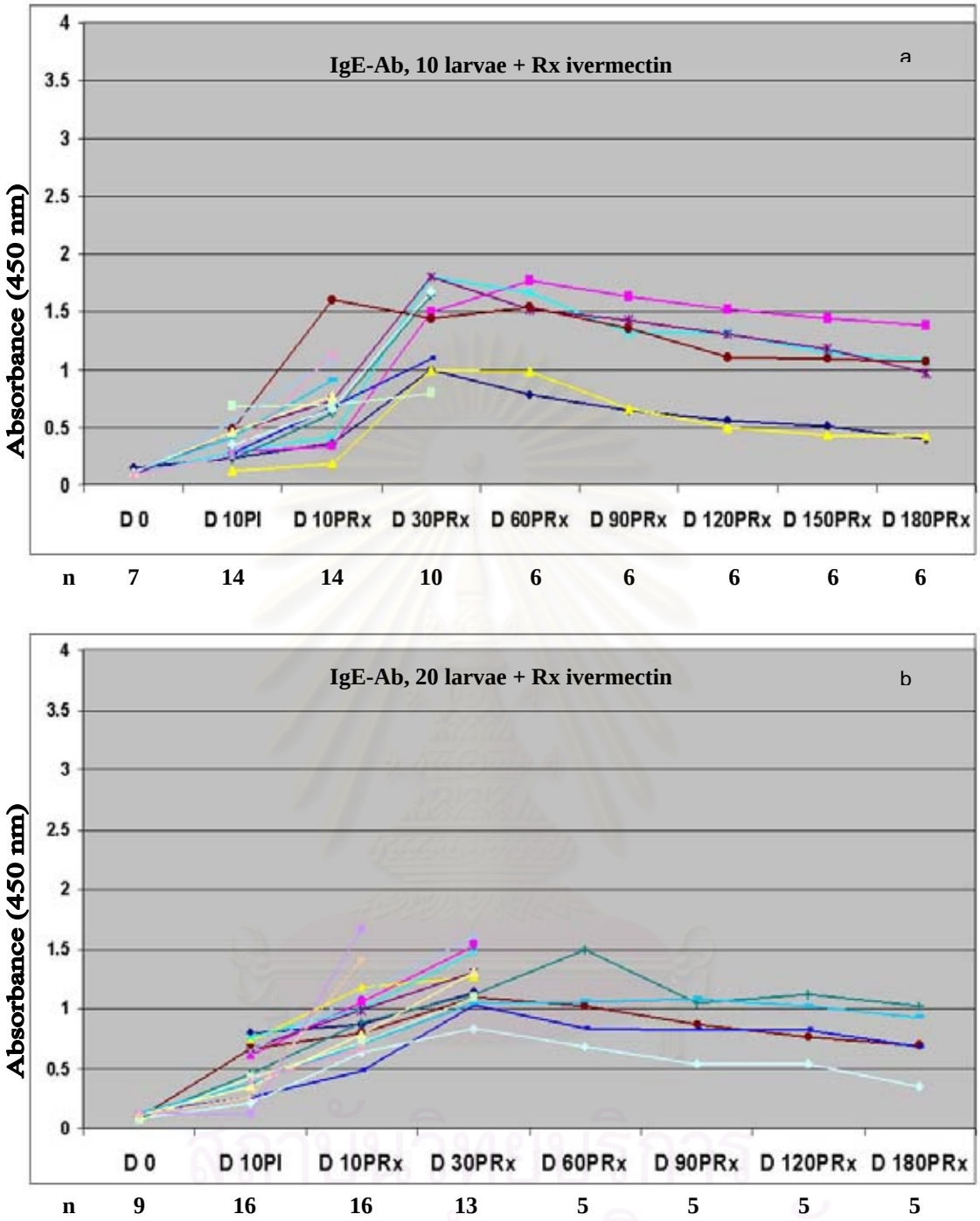


Fig 26. Kinetic of optical density of ELISA for IgE antibody to somatic L3s antigen in treated infected mice with 10 (a) and 20 (b) larvae of *G.spinigerum* at different time points (D 10, 30, 60, 90, 120, 150 and 180 PRx). Each line represents OD values of individual animal; n is the number of animal.

Table 5. Absorbance values of specific IgE antibody in serum of mice infected with *G.spinigerum* at different time intervals.

Group of mice		Absorbance density (450 nm)								
		D 0	D 10 PI	D 10 PRx	D 30 PI, PRx	D 60 PI, PRx	D 90 PI, PRx	D 120 PI, PRx	D 150 PI, PRx	D 180 PI, PRx
10 larvae	N	16	16		15	12	12	12	12	12
	X	0.160	0.481	-	1.127	1.437	1.486	1.413	1.410	1.181
	SD	0.079	0.196		0.349	0.310	0.488	0.419	0.472	0.652
20 larvae	N	9	9		8	5	5	5	5	5
	X	0.139	0.554	-	1.289	1.315	1.340	1.291	1.229	1.084
	SD	0.057	0.257		0.337	0.214	0.179	0.149	0.148	0.107
10 larvae	N	7	14	14	10	6	6	6	6	6
+ Rx	X	0.106	0.363	0.728	1.373	1.377	1.174	1.050	0.967	0.889
ivermectin	SD	0.022	0.151	0.371	0.372	0.399	0.418	0.422	0.404	0.392
20 larvae	N	9	16	16	13	5	5	5		5
+ Rx	X	0.104	0.484	0.937	1.222	1.020	0.874	0.852	-	0.736
ivermectin	SD	0.023	0.217	0.303	0.223	0.303	0.215	0.226		0.261

N = Number tested

X = Mean of Absorbance density

SD = Standard deviation of the mean

PI = Post infection

PRx = Post drug treatment

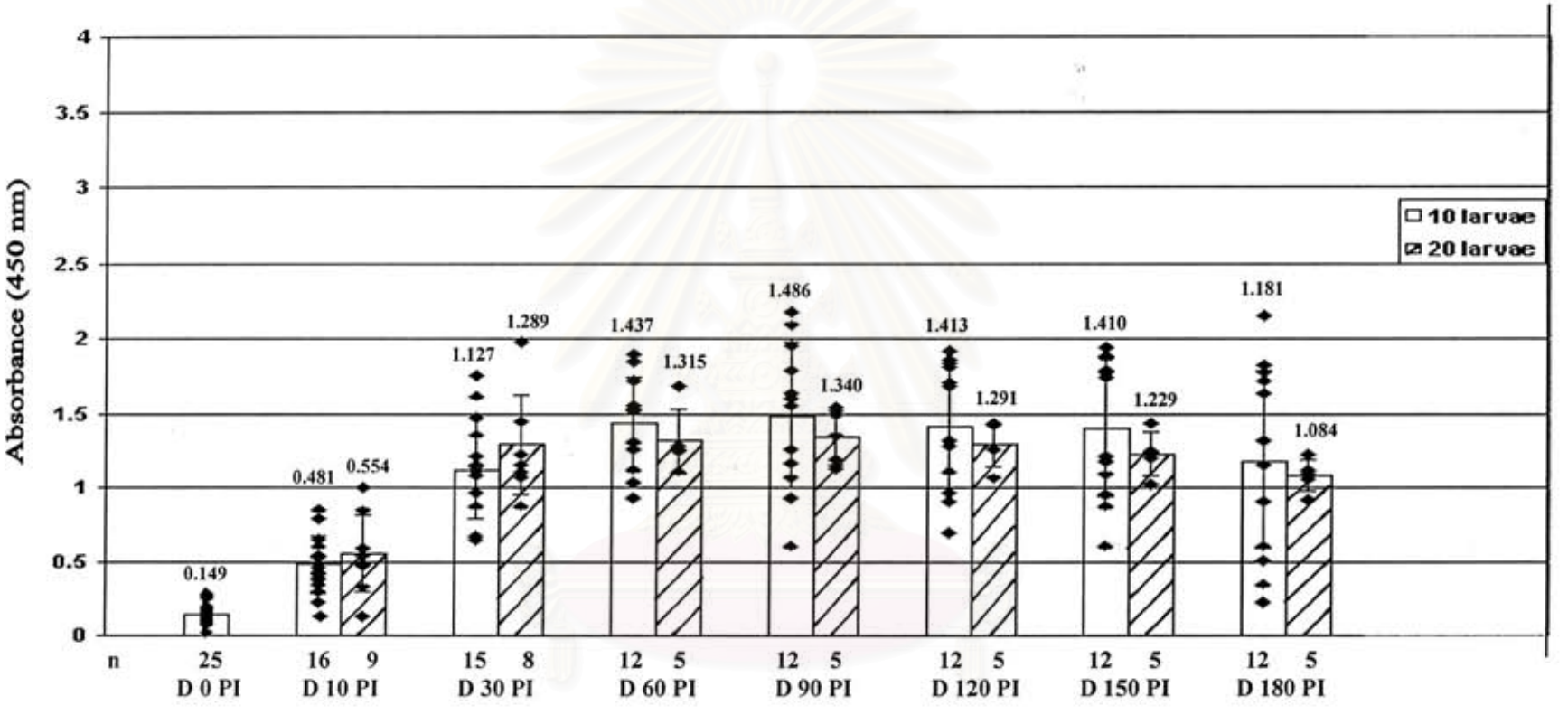


Fig 27. Optical density value of ELISA for IgE antibody to somatic L3s antigen in mice infected with 10 and 20 larvae of *G.spinigerum* at different time points (D 10, 30, 60, 90, 120, 150 and 180 PI). Bars and lines represent mean and SD of individual animal indicated (•); n is the number of animal.

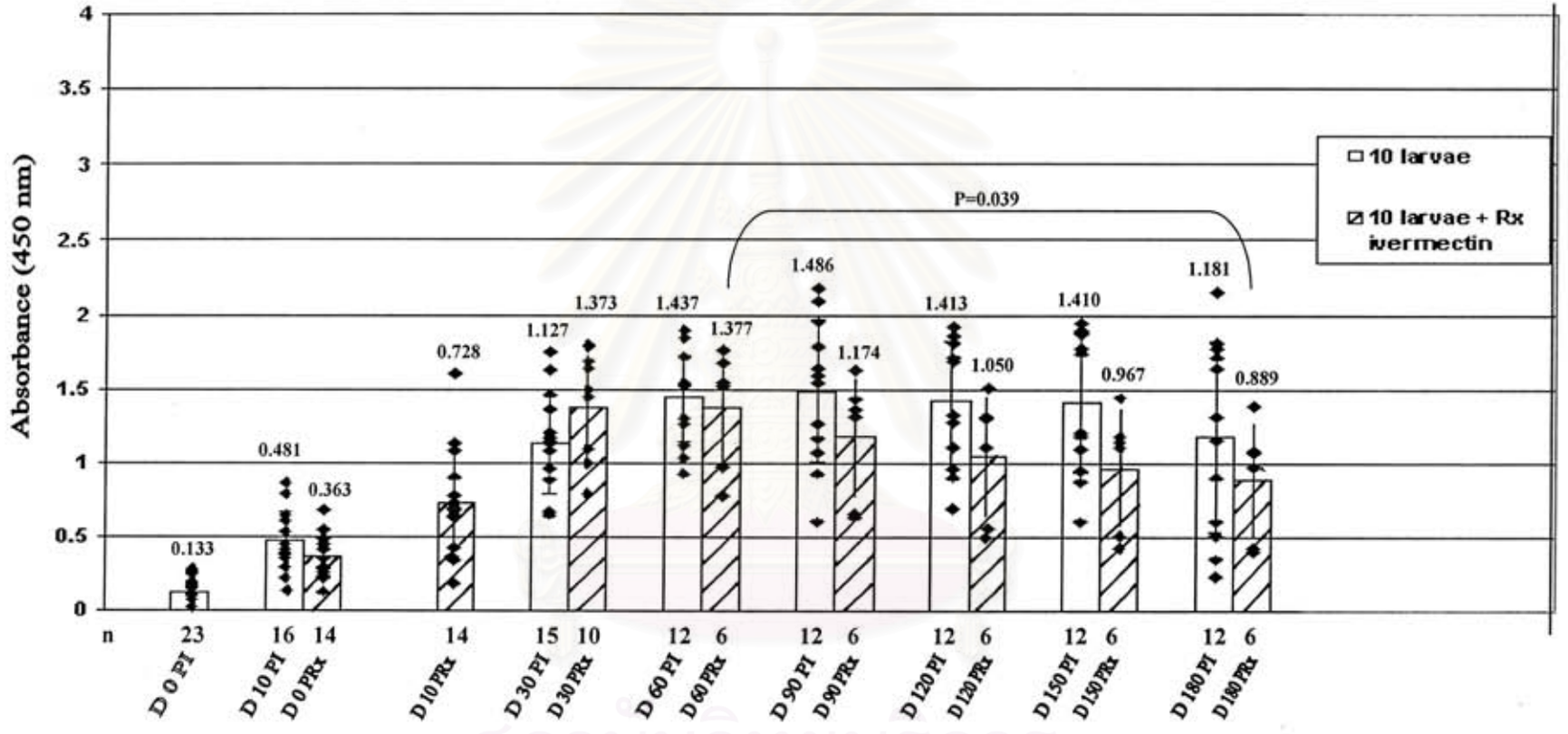


Fig 28. Optical density value of ELISA for IgE antibody to somatic L3s antigen in mice infected with 10 larvae and treated infected 10 larvae of *G.spinigerum* at different time points (D 10, 30, 60, 90, 120, 150 and 180 PI, PRx). Bars and lines represent mean and SD of individual animal indicated (•); n is the number of animal. PI: post infection; PRx: post drug treatment.

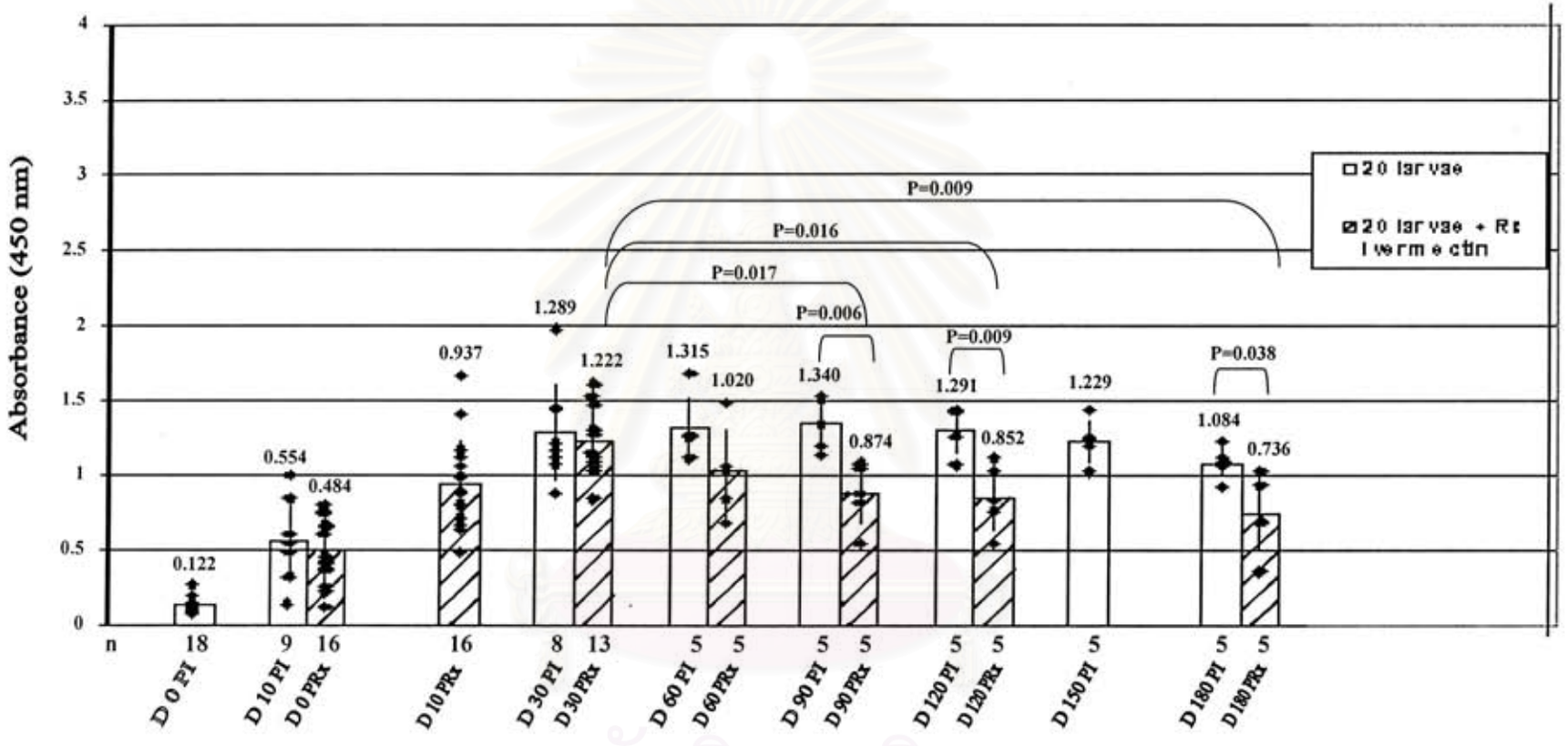


Fig 29. Optical density value of ELISA for IgE antibody to somatic L3s antigen in mice infected with 20 larvae and treated infected 20 larvae of *G.spinigerum* at different time points (D 10, 30, 60, 90, 120, 150 and 180 PI, PRx). Bars and lines represent mean and SD of individual animal indicated (•); n is the number of animal. PI: post infection; PRx: post drug treatment.

All the ELISA value of the studied group summarized in Table 2-5 and Fig 30-33 showed that IgG1, IgG and IgE were the three top dominant antibody of the infected sera while the IgG2a had minimal response. The mean IgG1 level showed about 21 times higher than the normal control. The IgG1-Ab of 10 larvae infected groups could demonstrate the statistically significant changes or decrease after drug treatment particularly those on Day 90 PRx and onward. Whereas the IgG-Ab of both 10 and 20 larvae infected mice persisted after being treated with anthelmintic drug through to the end of the experiment (6 months PRx, Fig 34 and Fig 35). In addition, similar to IgG1, IgE could demonstrate statistically significant changed after the infected mice were treated with drug (Fig 36 and Fig 37).

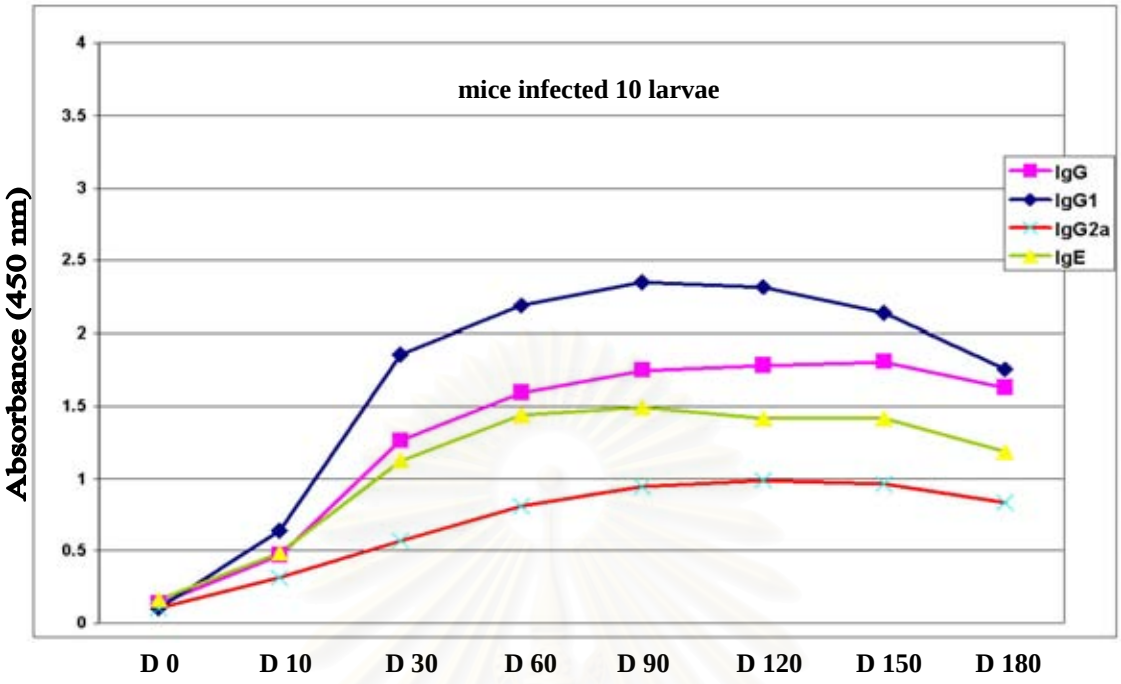


Fig 30. Summary of kinetic responses of IgG, IgG1, IgG2a and IgE-ELISA of mice infected with 10 larvae of advanced L3s *G.spinigerum*. Blood was taken from the infected mice at different time intervals (D 10, 30, 60, 90, 120, 150 and 180 PI).

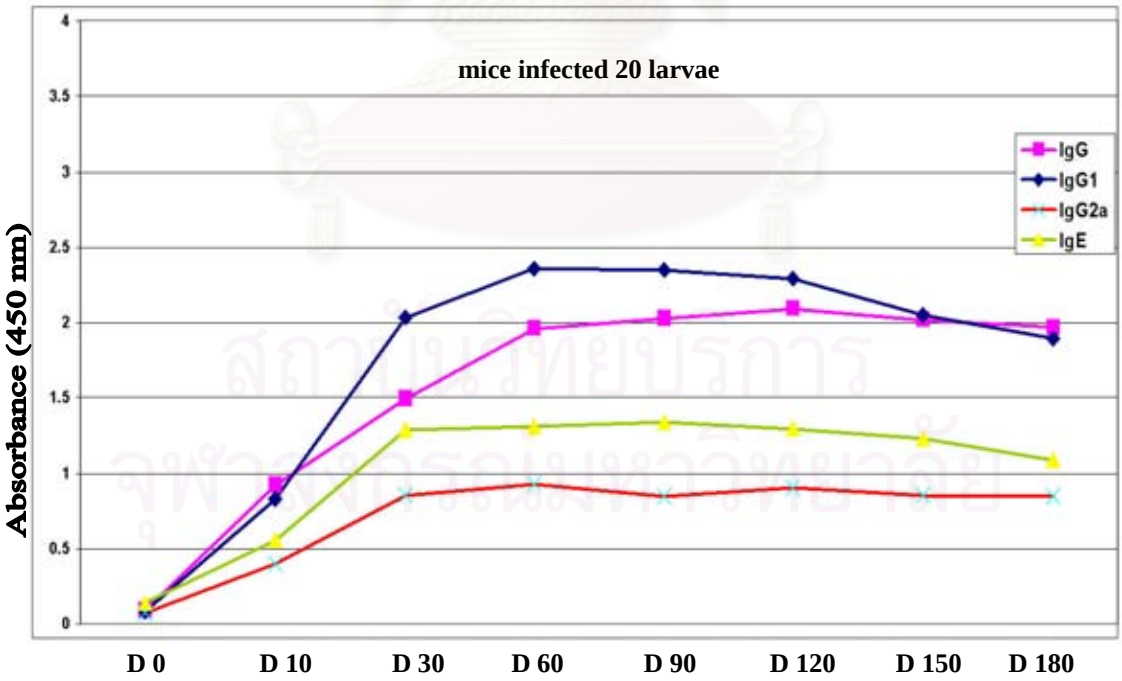


Fig 31. Summary of kinetic responses of IgG, IgG1, IgG2a and IgE-ELISA of mice infected with 20 larvae of advanced L3s *G.spinigerum*. Blood was taken from the infected mice at different time intervals (D 10, 30, 60, 90, 120, 150 and 180 PI).

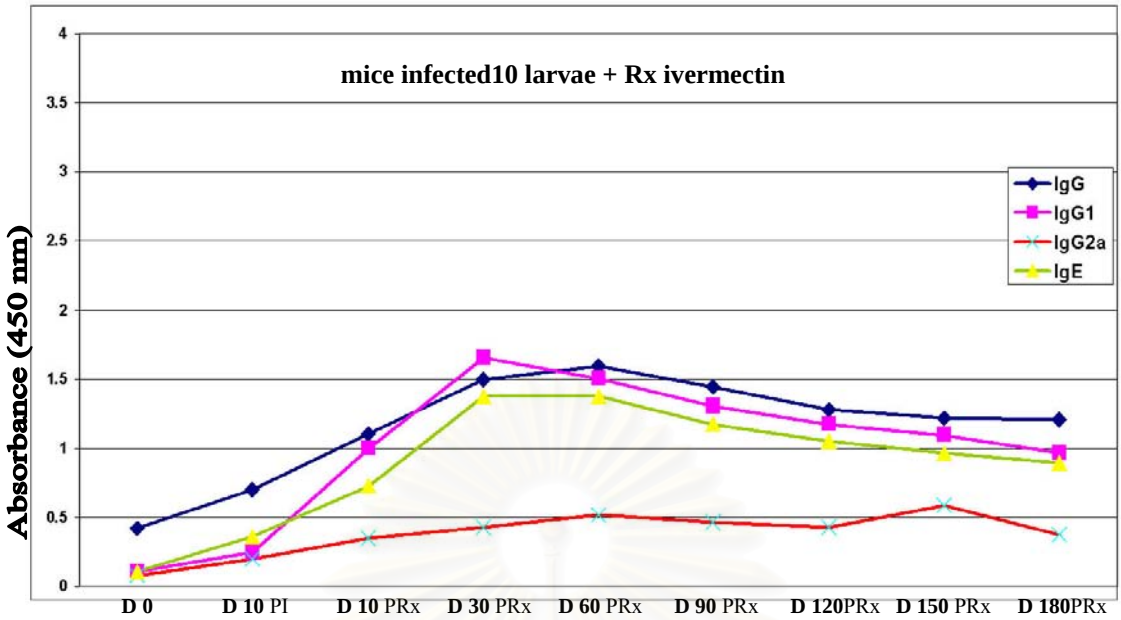


Fig 32. Summary of kinetic responses of IgG, IgG1, IgG2a and IgE-ELISA of treated infected mice with 10 larvae of advanced L3s *G.spinigerum*. Blood was taken from the infected mice at different time intervals (D 10, 30, 60, 90, 120, 150 and 180 PI).

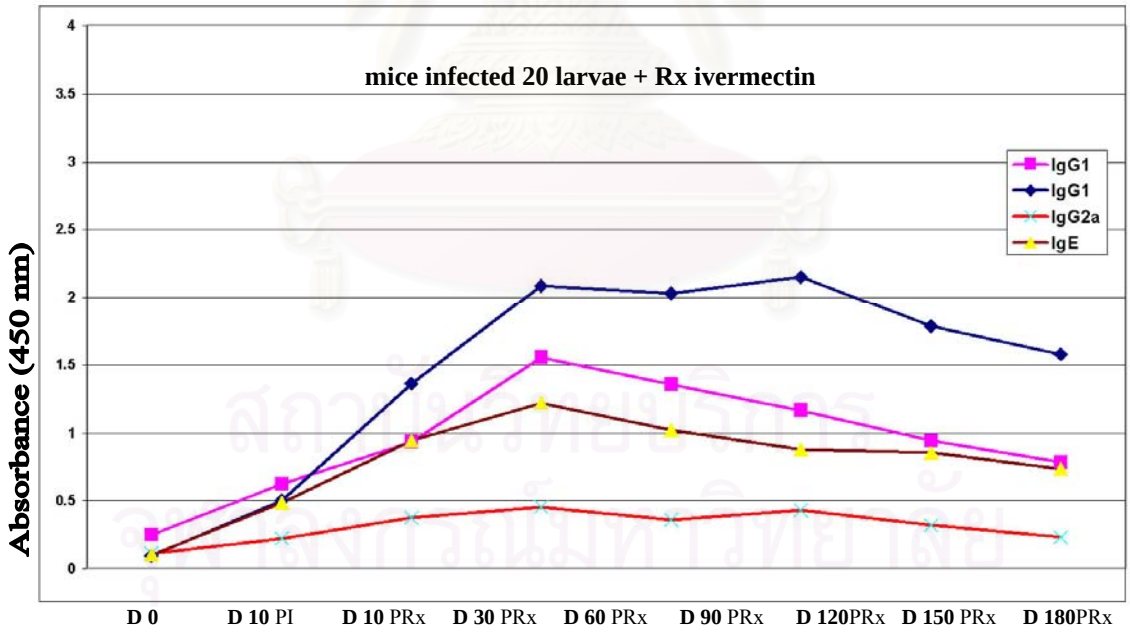


Fig 33. Summary of kinetic responses of IgG, IgG1, IgG2a and IgE-ELISA of treated infected mice with 20 larvae of advanced L3s *G.spinigerum*. Blood was taken from the infected mice at different time intervals (D 10, 30, 60, 90, 120, 150 and 180 PI).

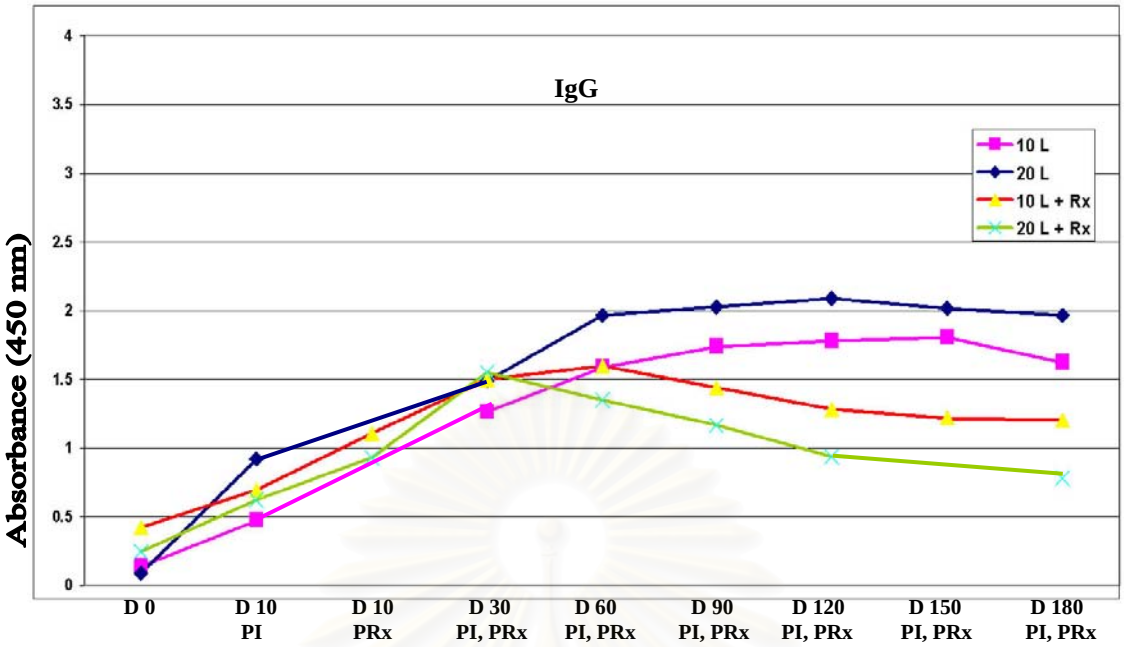


Fig 34. Means of IgG-ELISA of mice infected with 10 and 20 larvae of advanced L3s *G.spinigerum* both treated and non-treated with ivermectin. Blood was taken from the infected mice at different time intervals (D 10, 30, 60, 90, 120 and 180 PI, PRx).

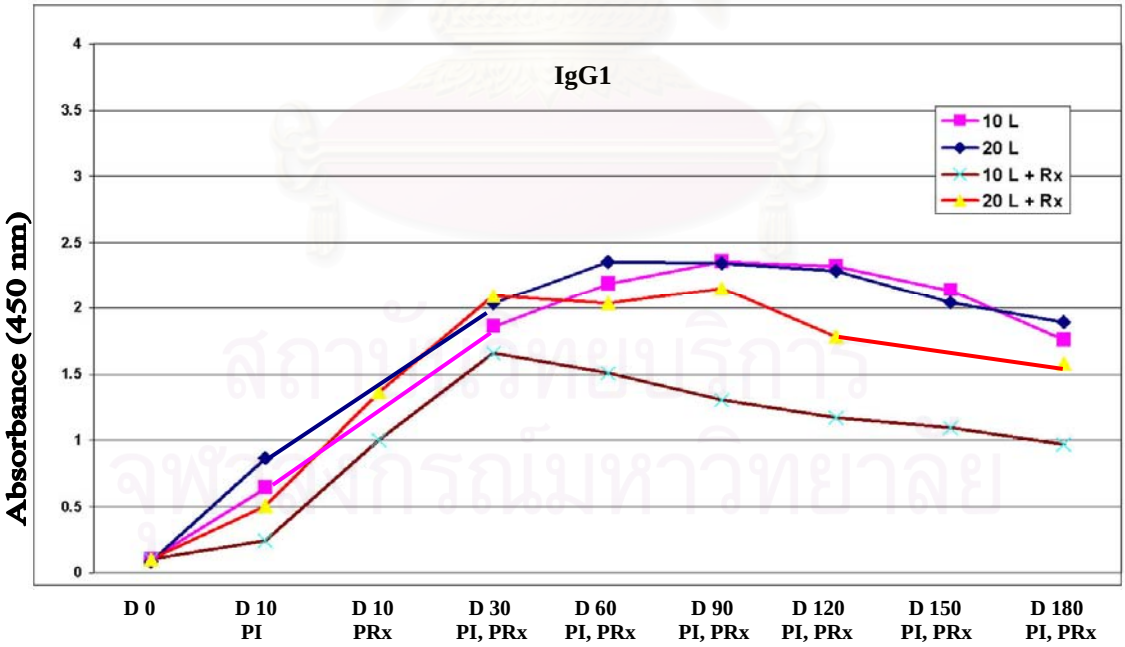


Fig 35. Means of IgG1-ELISA of mice infected with 10 and 20 larvae of advanced L3s *G.spinigerum* both treated and non-treated with ivermectin. Blood taken from infected mice at different time intervals (D 10, 30, 60, 90, 120 and 180 PI, PRx).

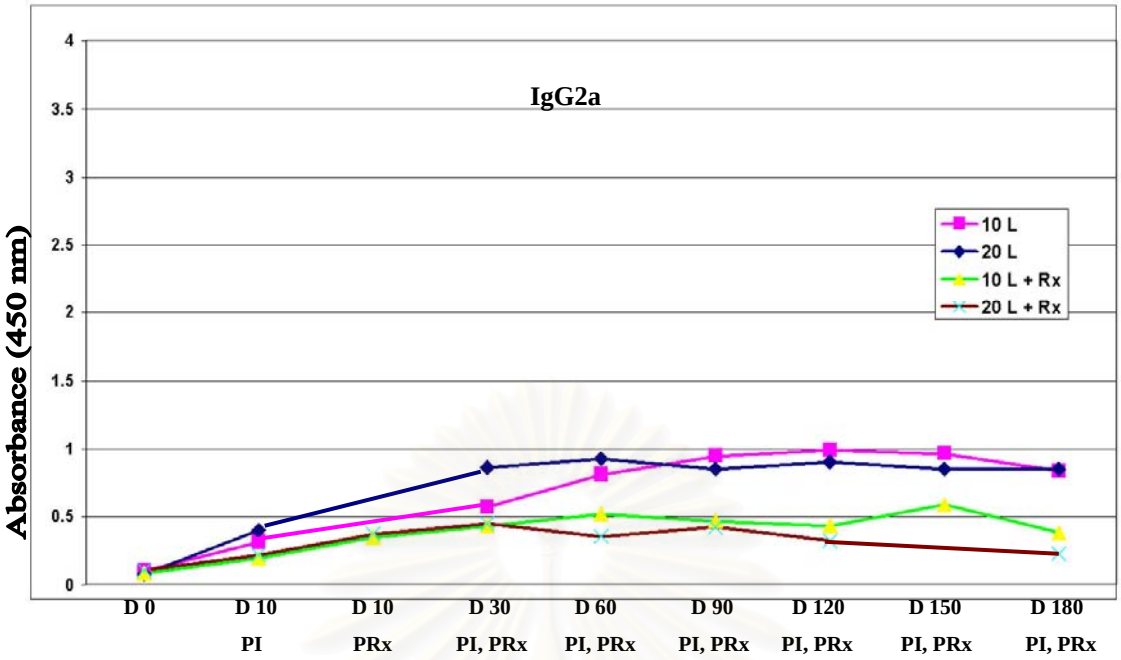


Fig 36. Means of IgG2a-ELISA of mice infected with 10 and 20 larvae of advanced L3s *G.spinigerum* both treated and non-treated with ivermectin. Blood was taken from infected mice at different time intervals (D 10, 30, 60, 90, 120 and 180 PI, PRx).

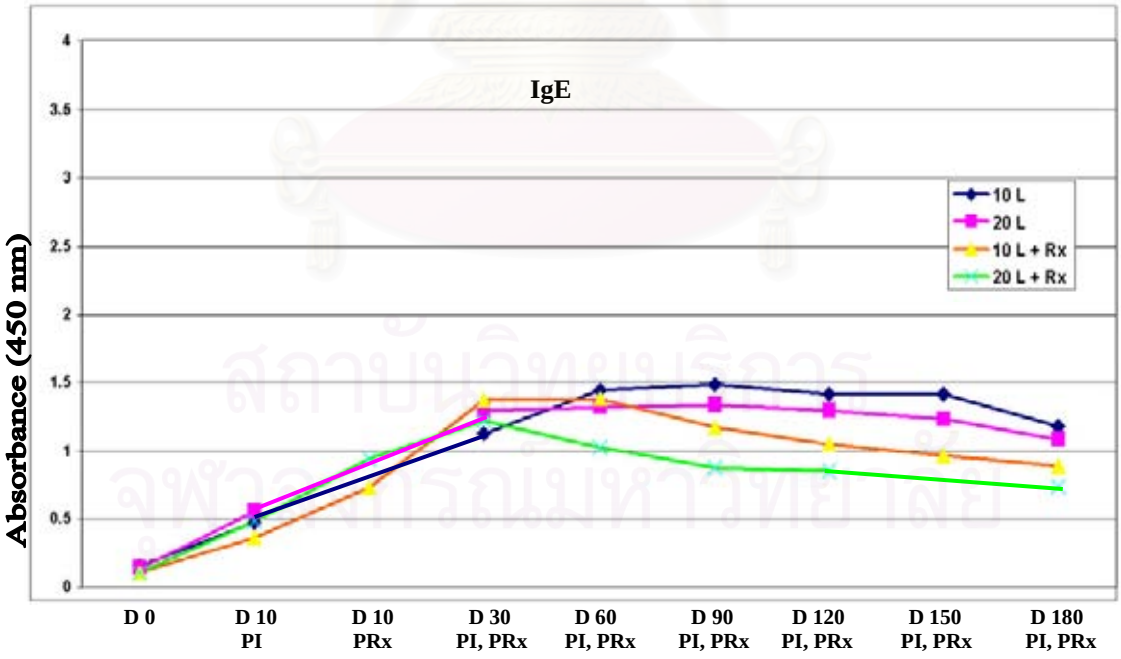


Fig 37. Means of IgE-ELISA of mice infected with 10 and 20 larvae of advanced L3s *G.spinigerum* both treated and non-treated with ivermectin. Blood was taken from infected mice at different time intervals (D 10, 30, 60, 90, 120 and 180 PI, PRx).

2. Lymphoproliferative response of mice infected with *G.spinigerum*

2.1 Determination of optimal conditions for Concanavalin A (Con A) - induced proliferative response of mouse splenic lymphocytes

Duration of stimulation and effect of Con A concentration

Lymphoproliferative response was set-up for mouse system using Con A as a mitogen. The procedure was performed as described with minor modification (Sciutto et al., 1995 and Kalra et al., 2002). Briefly, mice splenic cells were adjusted to a concentration of $2x10^6$ and $5x10^6$ cells/ml in RPMI 1640. One hundred microlitres of these splenocyte suspensions were triplicately cultured (37 ^{O}C, 5% CO_2) in microplate in the presence of Con A at concentrations of 1, 5 and 15 μg/ml in RPMI 1640 supplemented with 10% fetal bovine serum. After 48 hrs and 66 hrs of cultivation, ^{3}H-thymidine was added. The cells were harvested 6-24 hrs after pulsing. The radioactivity counted at $5x10^6$ cells/ml with 1 μg/ml of Con A provided the high stimulation index (Table 6).

Table 6 Effect of time of incubation and Con A concentration for mouse splenic lymphoproliferative response.

Number of cells (cells/ml)	Time of incubation (hr)*, #	Number of mice	Stimulation index (SI)		
			Con A concentration (μg/ml)		
			1	5	15
$2x10^6$	48, 18	4	11.07	13.54	3.58
	48, 24	1	-	8.02	-
	66, 6	1	-	23.48	-
$5x10^6$	48, 18	2	20.16	13.18	8.19
	48, 24	1	12.21	-	-
	66, 6	1	6.65	-	-

* , Incubation time prior to the addition of ^{3}H-thymidine

, Incubation time after pulsing to cell harvesting.

2.2 Effect of infection by advanced L3s *G.spinigerum* on lymphoproliferative response

In this experiment, mice were infected with 10 and 20 larvae of advanced L3s *G. spinigerum*. Splenic lymphoproliferative response to both non-specific mitogen (Con A) and specific antigen of somatic advanced L3s were determinant at 10, 50 and 180 days post infection, PI.

In addition, these responses were performed in the infected animals in infections were cured by oral administration of ivermectin.

2.2.1 Con A-stimulated lymphoproliferative response of *G.spinigerum* infected mice

Lymphocytes were taken from mice during the early-stage (10 and 50 days, PI) and the late-stage (180 days, PI) of infection. Their responses to Con A stimulation were compared with controls of the same age group.

2.2.1.1 Effect of early and late infection

The results of splenic lymphoproliferative responses of mice tested at 10, 50 and 180 days after infected with 10 and 20 larvae of advanced L3s summarised in Table 7. The splenic lymphocyte response of mice infected with 10 larvae at Day 10 was not significant from that of uninfected control (SI = 23.86 and 23.84). At day 50, the response was lower than that of Day 10, PI ($P = 0.023$). When the duration of infection with these 10 larvae prolong to the late stage (Day 180), a statistically significant low response was also noted ($P = 0.001$) compare to the group of Day 10 PI and the normal age match control mice. As shown in Table 7 and Fig 38, both the stimulation index and the absolute count of radioactivity (Δcpm) of the splenic lymphocyte from group of Day 50 and Day 180 PI were significantly (SI = 20.05 and SI = 18.03) lower than group of Day 10 PI (SI = 23.86) and than the uninfected controls (SI = 23.84). Since, the responses of uninfected controls mice with age of about 1-3 months and those with age of 7-8 months were not different, therefore ages had not affected.

When the degree of infection was increase by infection with 20 larvae, lower lymphoproliferative responses of Con A were also noted as shown in Table 7 and Fig 38. When the mice were infected for 10 days, the response in this group (SI = 21.98)

tended to decrease but was not statistically significant from that of uninfected control (SI = 23.86). At Day 50 PI, the response was lower than that of uninfected control (P = 0.009), but not significant lower than that of the groups of Day 10 PI (SI = 19.08 and 21.98). At Day 180, a marked reduction could be noted. The response of this group was lower or showed more unresponsed than those infected for a shorter duration. Comparing dose of infection, it was found that, the response of infection with 20 larvae of *G.spinigerum* (SI = 21.98, 19.08 and 17.57) tended to lower than that of infection with 10 larvae (SI = 23.86, 20.05 and 18.03) at the same time intervals of infection. However, it was not significantly different.

2.2.1.2 Effect of previous exposure to *G.spinigerum*

The above result showed significant depression of lymphoproliferative response (Day 50 and Day 180). It is therefore interesting to find out whether such degree of depression is reversible. We perform the test by treatment with a single oral dose of ivermectin at 0.4 mg/kg body weight.

In the experiment, mice infected with 10 and 20 larvae for 10-12 days, were treated with ivermectin orally. The first group of animal for testing the lymphoproliferative response was those sacrified at 10 days after treatment. Results presented in Table 7 and Fig 39, 40 showed that when the ivermectin treated mice with 10 larvae, the response was slightly higher than that of uninfected controls at all time point after treatment (10, 50 and 180 days). When the severity of infection was increased by infection with 20 larvae as shown in Fig 40, groups of mice were treated for 10 and 50 days, the response in this group was not significant from that of uninfected controls (SI = 22.24, 23.52 and 23.84). When the duration infection with these 20 larvae of advanced L3s prolong to the chronic stage (180 days), a statistically significant higher response was noted (SI = 29.14) compare to the group of 10 and 50 days post treatment and the uninfected controls (P = 0.000, 0.001 and 0.001).

As show in Fig 39, 40 that the mean value of SI and Δcpm of group of non-treated infected mice compare to group of treated infected mice, it was found that the response was not significant for acute infection (10 days) both infected with 10 and 20 larvae. When duration of infection was prolonged to day 50 and 180, the response of infected treated mice group was higher than in those non-treated infected groups.

It should be mentioned that prior to the drug treatment, the lymphoproliferative response of these animals was already somewhat unresponsive or depressed. Data from Table 7 and Fig 39 showed 10 days after treatment, the lymphoproliferative response to Con A stimulation of 20 larvae infected group was still depressed. Interestingly, there was an increase in response of 50 days and 180 days interval after treatment. The responses were enhanced when compared to the non-treated group. The mean stimulation indexes were 23.52 and 29.14, which were significantly higher than the non-treated correspond mice of 50 days PI (SI = 19.08) and 180 days PI (SI = 17.57) respectively (Fig 39).

Table 7 Concanavalin A stimulated lymphocyte proliferative response in infected mice *G.spinigerum*

Group / Day after infection	No. of mice	Counts per minute (cpm)		SI (S/U)	Δ cpm (S-U)
		Unstimulated (U)	Stimulated (S)		
Control	18	5,654.52 ± 935.84*	133,844.49 ± 27,042.15	23.84 ± 3.78	128,123.22 ± 26,531.68
10 larvae					
10 days	4	4,520.02 ± 490.13	107,309.90 ± 6,097.93	23.86 ± 1.62	102,789.89 ± 5,666.82
50 days	6	5,787.66 ± 885.49	114,777.17 ± 14,422.80	20.05 ± 2.67	108,989.51 ± 13,949.82
180 days	12	5,951.95 ± 965.45	105,387.37 ± 14,924.57	18.03 ± 3.25	99,435.42 ± 14,769.03
20 larvae					
10 days	4	4,990.96 ± 354.48	109,511.59 ± 7,352.49	21.98 ± 1.32	104,520.63 ± 7,131.55
50 days	5	5,922.14 ± 979.04	112,519.64 ± 23,537.77	19.08 ± 2.61	106,597.51 ± 22,838.38
180 days	5	5,733.00 ± 331.67	102,692.97 ± 6,957.13	17.57 ± 1.13	94,959.98 ± 8,304.00
10 larvae + Rx					
10 days	5	4,659.36 ± 470.94	111,420.77 ± 9,070.87	23.97 ± 1.01	106,761.41 ± 8,640.62
50 days	6	5,835.69 ± 1,398.74	146,338.75 ± 29,484.72	25.36 ± 3.06	140,503.06 ± 28,250.48
180 days	6	4,434.20 ± 257.97	114,635.51 ± 5,662.60	25.59 ± 2.40	110,201.32 ± 5,752.16
20 larvae + Rx					
10 days	4	5,132.39 ± 590.33	113,738.85 ± 9,529.52	22.24 ± 1.28	108,606.47 ± 9,019.18
50 days	8	6,507.44 ± 761.09	154,057.74 ± 31,286.46	23.52 ± 2.73	147,550.29 ± 30,634.37
180 days	5	6,721.42 ± 851.71	190,581.46 ± 11,004.37	28.59 ± 2.48	183,860.04 ± 10,285.04

* Mean ± SD

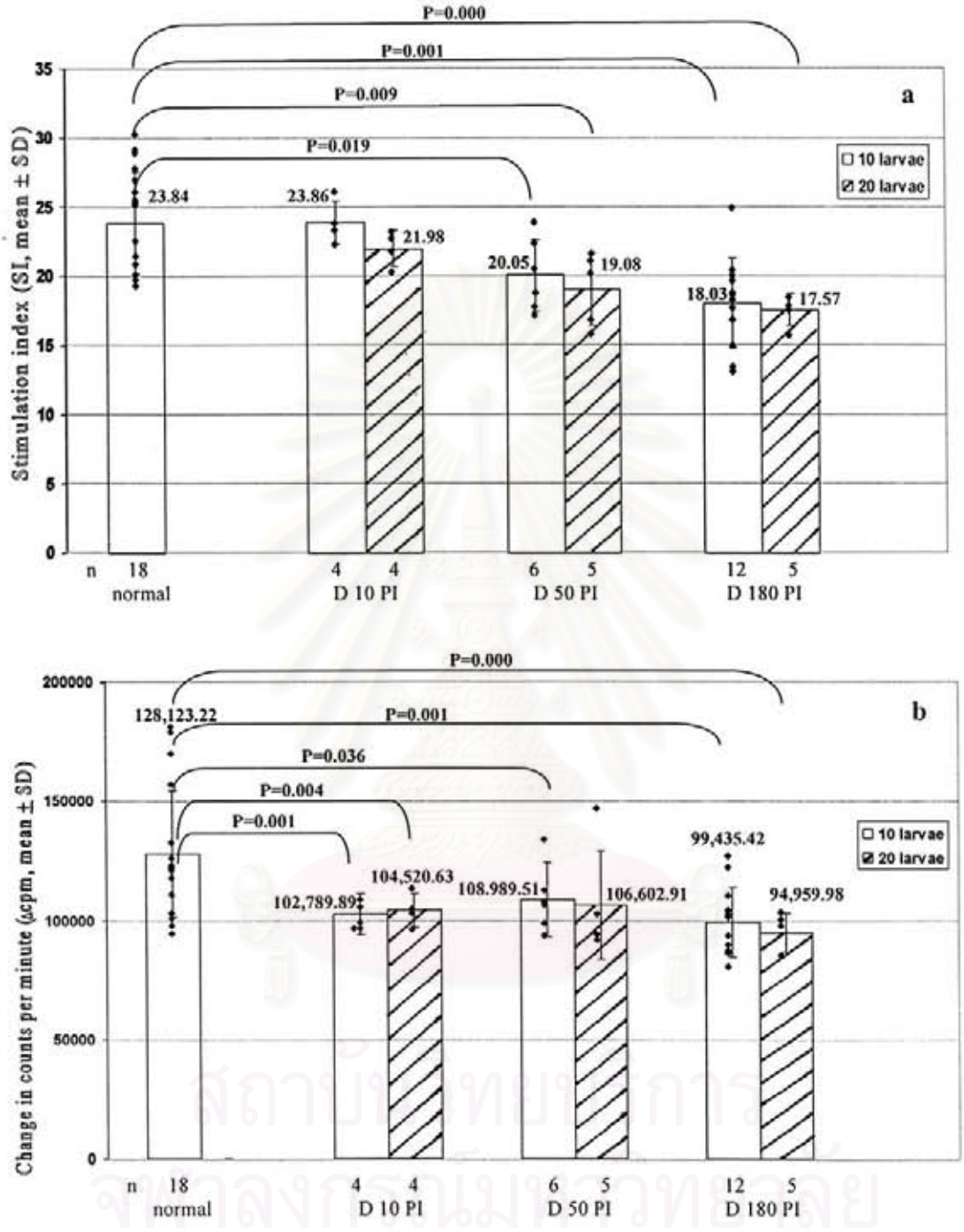


Fig 38. Stimulation index (SI, a) and change in counts per minute (Δcpm, b) of Con A-stimulated splenic lymphocytes of mice infected with 10 and 20 larvae of *G.spinigerum* at different time intervals (D 10, 50 and 180). Bars and lines represent mean and SD of individual animal indicated (•); n is the number of animal in each experimental group. PI is post infection.

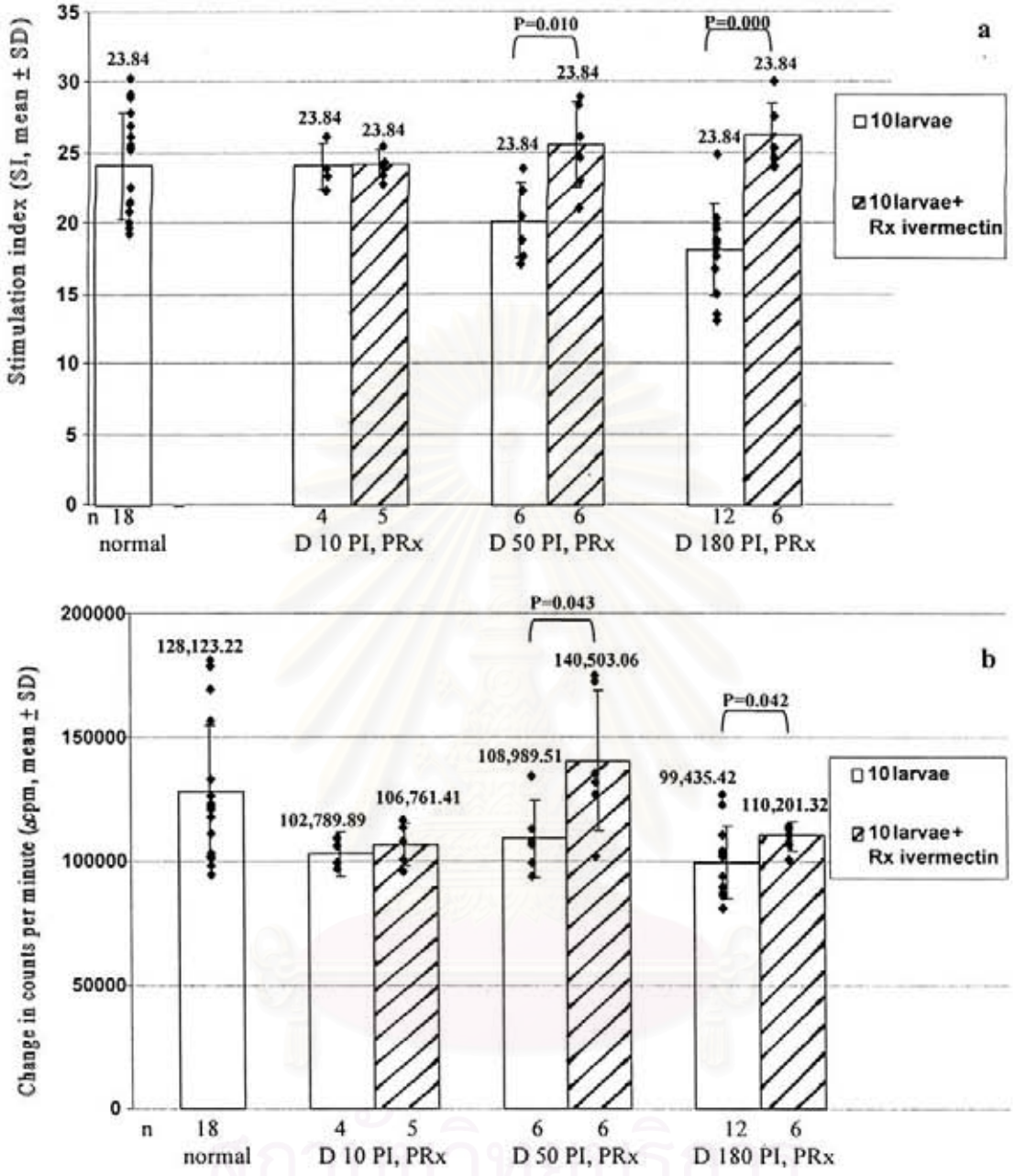


Fig 39. Stimulation index (SI, a) and change in counts per minute (Δcpm, b) of Con A-stimulated splenic lymphocytes of mice infected with 10 larvae and treated infected 10 larvae of *G.spinigerum* at different time intervals (D 10, 50 and 180). Bars and lines represent mean and SD of individual animal indicated (•); n is the number of animal in each experimental group. PI is post infection; PRx is post drug treatment.

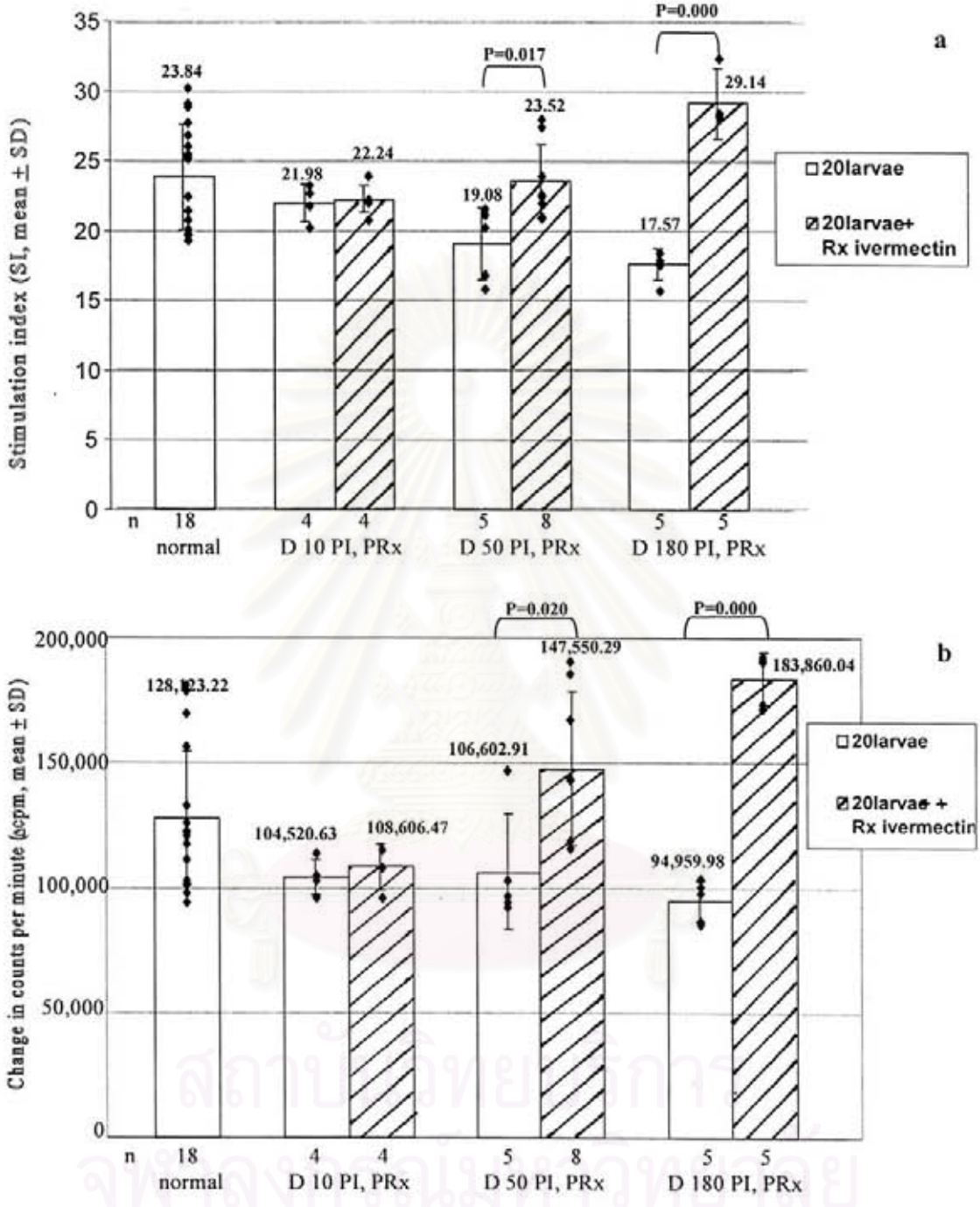


Fig 40. Stimulation index (SI, a) and change in counts per minute (Δcpm, b) of Con A-stimulated splenic lymphocytes of mice infected with 20 larvae and treated infected 20 larvae of *G.spinigerum* at different time intervals (D 10, 50 and 180). Bars and lines represent mean and SD of individual animal indicated (•); n is the number of animal in each experimental group. PI is post infection; PRx is post drug treatment.

2.2.2 Antigen-stimulated Lymphoproliferative response of mouse infected with L3s *G.spinigerum*

2.2.2.1 Effect of early and late infection

In this experiment, a specific response to somatic antigens was determined. Lymphocytes taken from mice during the early-stage (10 and 50 days) and the late-stage (180 days) of infection were tested with 100 μg/ml somatic L3s *G.spinigerum* antigen. The responses were compared with controls of the same age group. The uninfected controls groups responded slightly to antigen stimulation, with a mean stimulation index of 0.97 (Table 8). Ten days after being infected with 10 and 20 larvae, there was a slight increase in response to antigen stimulation (SI = 1.47 and SI = 1.12). It was statistically significant (P = 0.000) between the group infected with 10 larvae and the uninfected controls group. Among these experimental animals, those at 50 days after being infected with 10 and 20 larvae had the highest stimulation index (SI = 2.34 and SI = 2.38) as shown in Fig 41. When the infected animals of 10 and 20 larvae were prolonged to 180 days, the stimulation indices were not as high as the group of 50 days PI. However, they were still significantly higher than the group of day 10 PI and uninfected controls. Most of the infected mice, the stimulation index of somatic L3s antigen stimulation was significantly higher than uninfected control group.

2.2.2.2 Effect of previous exposure to *G.spinigerum*

Prior to single dose of ivermectin (0.4 mg/kg body weight) giving, animals was given to animals. Results summarized in Table 8 and Fig 42 and 43 showed that the response of both infected 10 and 20 larvae was gradually increase following days post treatment ($P<0.05$). For acute infection (10 and 50 days) the response was significant higher than uninfected controls. For chronic infection (180 days) the response a significant high response was noted ($P<0.05$) compare to the group of 10 and 50 days post treatment and the uninfected controls. The interesting observation however was the finding that 180 days after treated with ivermectin, the lymphocyte responses were markedly enhanced. The mean stimulation index was more than about 8 times higher than that of the control groups. The response of this group was higher than in those treated for a shorter duration.

The comparison of stimulation index between non-treated infected mice and treated infected mice was showed in Fig 42-43, both the SI and Δcpm. The interesting observation was the finding that 10, 50 and 180 days after the infection were cured by ivermectin treatment, the lymphoproliferative responses were markedly enhanced. The mean stimulation index was about 2 times than that of the non-treated mice in group of day 10 and day 50 after being treated with chemotherapy. At the late stage of infection (180 day), the mean stimulation index was approximately 4 times higher than that of the non-treated group. At same days of post treatment, it was showed that the response of treated infected mice was statistically significant higher than non-treated infected mice in all group at both 50 and 180 day PRx.

The results presented in this study showed that splenic lymphocytes from mice infected with both 10 and 20 larvae of *G.spinigerum* had altered reactivity to stimulation by Con A and depressed response to specific stimulation by somatic L3s antigen.

Table 8 Antigen- stimulated lymphocyte proliferative response in infected mice *G.spinigerum*

Group / Day after infection	No. of mice	Counts per minute (cpm)		SI (S/U)	Δ cpm (S-U)
		Unstimulated (U)	Stimulated (S)		
Control	18	4,712.19 ± 1,896.15*	4,554.37 ± 1,958.93	0.97 ± 0.18	-157.82 ± 995.88
10 larvae					
10 days	4	4,538.81 ± 439.24	6,660.26 ± 541.50	1.47 ± 0.09	2,121.45 ± 362.03
50 days	6	4,796.92 ± 537.49	11,112.25 ± 314.60	2.34 ± 0.25	6,315.33 ± 473.87
180 days	12	4,265.67 ± 1,273.21	8,163.17 ± 1,688.47	1.99 ± 0.39	3,897.50 ± 1,310.36
20 larvae					
10 days	4	4,325.31 ± 668.33	4,796.16 ± 214.56	1.12 ± 0.14	470.86 ± 463.36
50 days	5	2,116.03 ± 254.71	4,941.35 ± 1,112.98	2.38 ± 0.70	2,825.32 ± 1,200.37
180 days	5	4,886.66 ± 1,216.66	7,845.84 ± 1,998.70	1.63 ± 0.40	2,959.17 ± 1,786.37
10 larvae + Rx					
10 days	5	5,753.05 ± 1,659.72	15,272.11 + 4,294.18	2.67 ± 0.25	9,519.06 ± 2,783.37
50 days	6	5,981.59 ± 1,507.18	30,015.56 ± 6,184.03	5.13 ± 0.68	24,033.97 ± 4,882.34
180 days	6	5,311.79 ± 642.14	45,819.41 ± 4,007.19	8.78 ± 1.71	40,507.62 ± 4,320.23
20 larvae + Rx					
10 days	4	4,79.53 ± 279.07	12,107.78 ± 224.88	2.56 ± 0.12	7,358.25 ± 212.50
50 days	8	5,507.55 ± 580.76	20,091.83 ± 3,144.66	3.62 ± 0.72	14,209.28 ± 3,459.23
180 days	5	4,282.25 ± 1,534.11	29,599.81 ± 7,778.08	7.68 ± 2.86	25,317.56 ± 6,918.11

* Mean ± SD

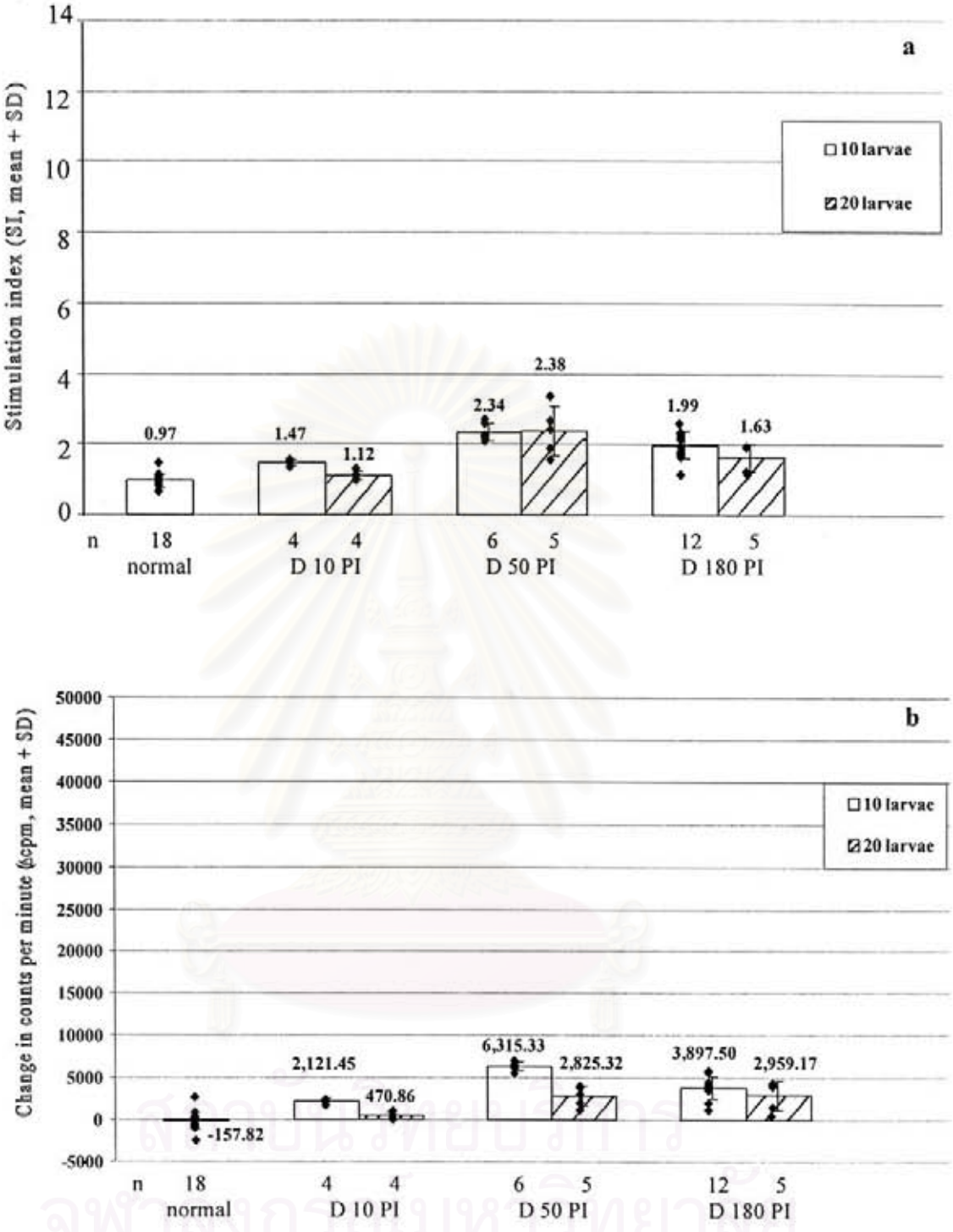


Fig 41. Stimulation index (SI, a) and change in counts per minute (Δcpm, b) of somatic L3s antigen stimulated splenic lymphocytes of mice infected with 10 and 20 larvae of *G.spinigerum* at different time intervals (D 10, 50 and 180). Bars and lines represent mean and SD of individual animal indicated (•); n is the number of animal in each experimental group. PI is post infection.

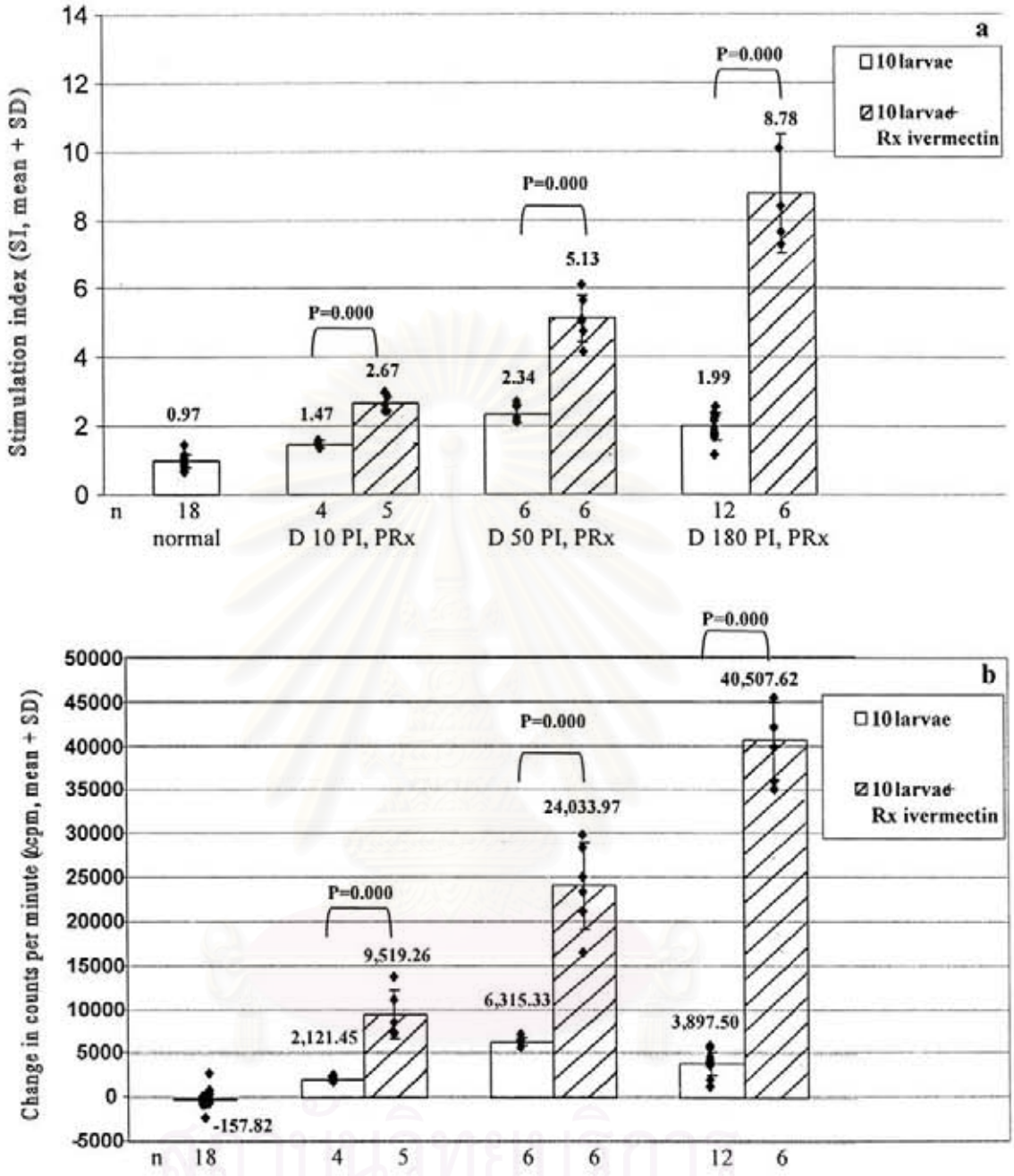


Fig 42. Stimulation index (SI, a) and change in counts per minute (Δcpm, b) of somatic L3s antigen stimulated splenic lymphocytes of mice infected with 10 larvae and treated infected 10 larvae of *G.spinigerum* at different time intervals (D 10, 50 and 180). Bars and lines represent mean and SD of individual animal indicated (•); n is the number of animal in each experimental group. PI is post infection; PRx is post drug treatment.

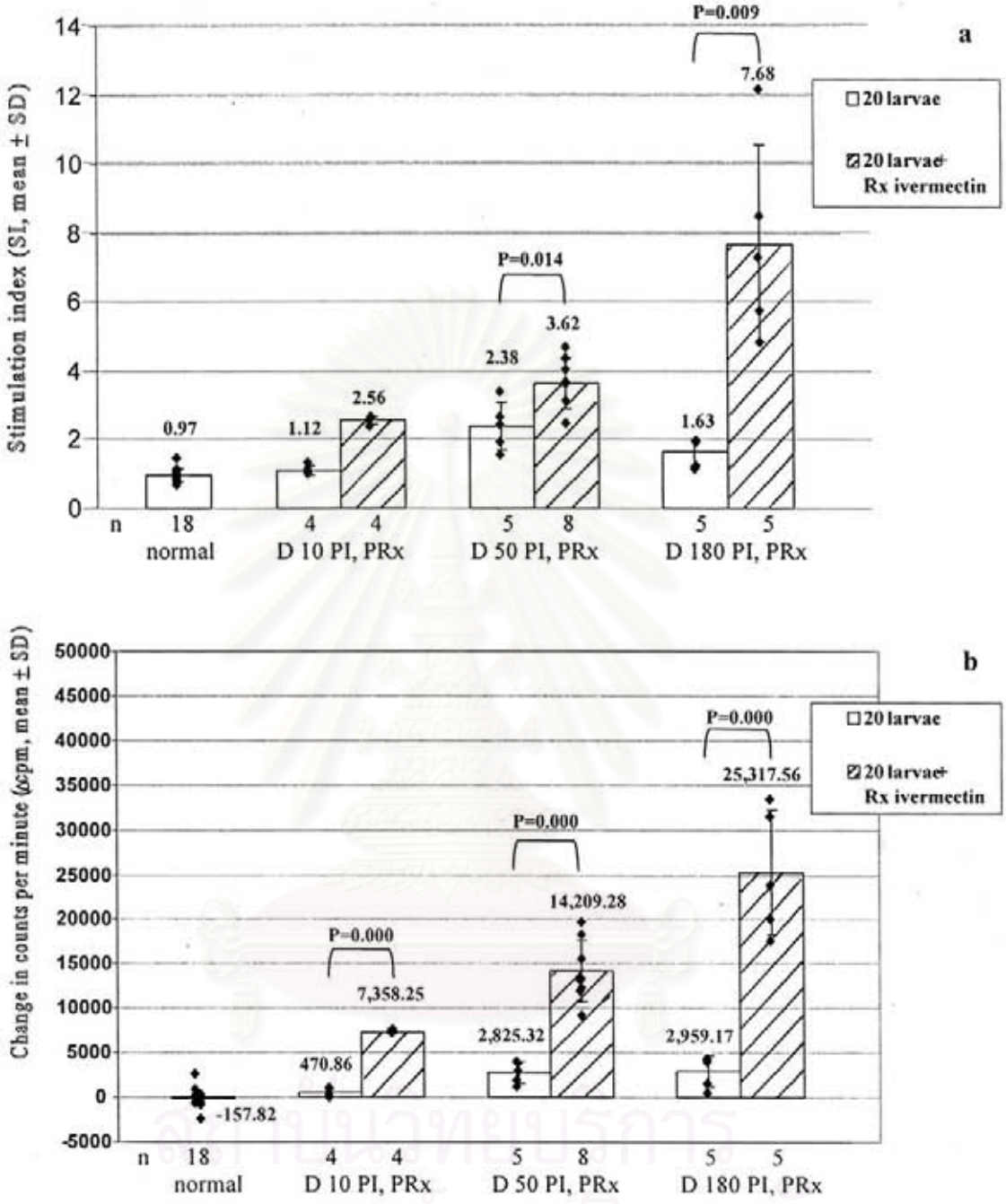


Fig 43. Stimulation index (SI, a) and change in counts per minute (Δcpm, b) of somatic L3s antigen stimulated splenic lymphocytes of mice infected with 20 larvae and treated infected 20 larvae of *G.spinigerum* at different time intervals (D 10, 50 and 180). Bars and lines represent mean and SD of individual animal indicated (•); n is the number of animal in each experimental group. PI is post infection; PRx is post drug treatment.

CHAPTER VI

DISCUSSION

In this experiment, the high infective dose was changed from the previous assignment of 30 larvae presented in the thesis proposal to 20 larvae. Since, in our preliminary study, all three mice infected with high dose of 30 larvae died. The first animal died on Day 2; the others died on Day 3 and Day 7 post infection respectively. Autopsy photos (belowed), showed severe trauma and disruption of liver parenchyma. In addition, infection with dose of 25 larvae was too high and resulted in dead of the infected animal as well. By these reasons we designed to infect the animal with 10 and 20 larvae. In addition, conditions that affected the experiment were as following:

1) Drop out of mouse from some group

2) The inadequate numbers of worm harvested from eels prolonged the time for animal infection.

3) Death of mice infected with 25, 30 larvae (too high) made the assigned dose of infection down to 20 larvae.

4) Optimal conditions for lymphoproliferative test could not be standardized until Day over 50 post infection. Therefore the 10 larvae infected mouse was accumulated to 12 mice on Day 180 post infection.

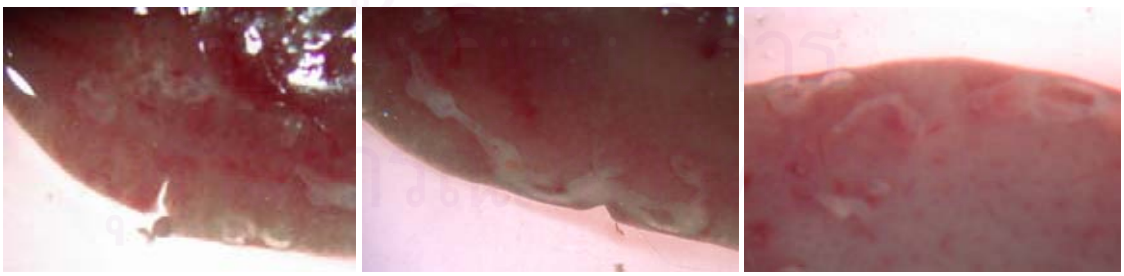


Fig 44. Severe trauma and disruption of liver parenchyma of mice infected with 30 larvae *G.spinigerum*.

1. Antibody responses of mice infected with *G.spinigerum*

In most cases of human gnathostomiasis, the worms obtained from surgery or from their spontaneous emergences were the third-stage larvae and immature adults (Daengsvang, 1980; Akahane et al., 1994; Kraivichian et al., 2004). And the antigens generally used for antibody detection should be prepared from the stage of parasite as that migrate in the host and is the first recognition stage by the host (Suntharasamai et al., 1985; Ada, 1993). Thus the third-stage larvae were selected for antigen in this study. The ELISA values of the previous study using antigen prepared from advanced L3s showed higher sensitivity and specificity when compared to those prepared from adult male and female (Morakote et al., 1991).

In this study, the ELISA using the advanced L3s somatic antigens could detect the antibody responses at different time intervals (Days 10, 30, 60, 90, 120 and 180 PI) of both groups of mice infected with 10 and 20 larvae. The increase of specific Ab (IgG-Ab, IgG1-Ab, IgG2a-Ab and IgE-Ab) was detected within Day 10 PI. The antibody levels was gradually increased and reached a plateau during Day 60 and Day 120 PI (Table 2-5). The Ab responses of the 10 larvae infected tended to lower than those of the 20 larvae infected animals. In heavy infection, Ab levels are usually higher than those in light infection (Abbas and Lichtman, 2003). From the overall kinetic responses, the predominant antibodies were IgG1-Ab, IgG-Ab and IgE-Ab. After reaching a plateau, all the detected Ab levels began to decrease approximately during Day 90 PI and Day 150 PI. The decreased antibody levels might be consisting with the time of the complement of larvae encystment. Since, the larvae encystment in infected mice firstly appeared or began at Day 11 PI, and about half of the larvae encysted Day 14 and Day 21 PI. All the larvae could completely encyst during Day 30 and Day 60 PI (Saksirisampant, personal communication).

The ELISA of IgG-Ab reached the peak at Day 120 PI in both infected groups. Following 6 months after ivermectin administration, the IgG-Ab level could persist or still be detected. During this period (6 months after being treated), the average worm recovery showed 11.66% of living parasites left in 10 larvae infected mice and showed 22.00% left in 20 larvae infected mice (Chawengkirttikul, personal communication).

This persisting antibody showed corresponding with the IgG-Ab level in human gnathostomiasis. Since, IgG-Ab responses were used as immunodiagnosis

tool or as an evidence of infection could still be detectable for more than six months following treatment with anthelmintic drug (Kraivichian et al., 1992; Nontasut et al., 2000). These facts should be taken into consideration in the interpretation of serological results, since a positive reaction may reflect a previous or a present infection.

With regard to the IgG1-Ab, it had highest response when compared to another subclass or isotype. It reached a maximum on Day 90 PI and 60 PI in 10 and 20 larvae infected mice respectively. Following ivermectin treatment, the level of IgG1-Ab in 10 larvae infected group could slight continually increase to Day 30 PRx but drastically decreased at Day 90 PRx and statistically significantly lower when compared to the corresponding non-treated mice (P=0.008). However, IgG1 level in 20 larvae infected group tended to decrease but had no significant difference from the corresponding non-treatment mice. However, it could show significant lower at Day 180 PRx. The slow decrease of this IgG1 during Day 90-180 PRx of the 20 larvae infected mice might be due to the high dose (20 larvae) of infection. The used-dose of drug efficiency might be more affected to the low dose of worms or light infection rather than that of high dose or heavy infection. Since the dose of ivermectin used at 0.4 mg/kg body weight demonstrated the mean living worm recovery at 11.66% of 10 larvae infected group while demonstrated 22.00% in 20 larvae infected group (Chawengkirttikul, personal communication).

The IgG2a showed low response in both 10 and 20 larvae infected groups. Following ivermectin treatment, there was no significant change of IgG2a-Ab which can indicate that Th-1 response might be inhibited.

The study of splenic lymphocyte proliferation in infected mice show a significant depression of the cellular response to Con A and somatic Ag. The depression was more severe with prolonged and heavy infection; suggest that the Th-1 cell response is defective (Table 7 and 8). These results were reinforced by the low level of IgG2a antibody mentioned previously.

A previous evidence of human gnathostomiasis demonstrated that involvement of Th-2 response might be existing. Since, anti-*G.spinigerum* L3s IgG1-Ab and IgG4-Ab provided the highest Ab level, while IgG2-Ab had the lowest level (Nuchprayoon et al., 2003). Human IgG1 and IgG4 antibodies is in general the reflect of the induction of Th-2 responses, whereas IgG2-Ab is that of an induction of Th-1 responses (Else and Grencis, 1991).

Furthermore, IgE-Ab in this study showed high response and reached a plateau on Day 90 PI. In 10 and 20 larvae with anthelmintic treated group, a significant decreased of the IgE-Ab could demonstrated at Day 180 and Day 90 PRx. *G.spinigerum* does not reach maturity in man. After the advanced L3s being ingested, these larvae will migrate through the intestinal wall to several tissues. An increase in parasite-specific IgE antibody is a particular feature of those parasites having a tissue migration. The significant high levels of IgE antibody in patients were reported in previous studies (Soesatyo et al., 1987; Saksirisampant et al., 2001). In addition, a significant decrease of this IgE-Ab specific to ES Ag had been demonstrated within 3 months of post albendazole treated patients (Saksirisampant, personal communication). However, a study of IgE-Ab response from post ivermectin treatment of both infected human and animals have never been reported yet. The high level of IgE-Ab is associated with the Th-2 response in many parasitic infections. Since, Th-2 cells can secrete IL-4, which is essential for IgE production (Romagnani S, 1991).

Many parasitic infections such as *Trichuris trichiura*, *Enterobius vermicularis*, *Ascaris lumbricoides*, *Strongyloides stercolaris* and *Brugia malayi* could induce a concomitant increase of the IgE and IgG1 serum levels and usually had low level of the IgG2a. This was due to the induction of IL-4 to the B cells to secrete both IgG1 and IgE (Romagnani, 1991; Mahanty et al., 1992 and Yazdanbakhsh et al., 1993).

Our results can support or help confirm the role of Th-2 response may involve in this mouse gnathostomiasis by the following reasons:

1) A higher level of IgG1-Ab could be demonstrated when compared to the IgG2a-Ab in both infected groups (Fig 30-33).

2) IgG2a-Ab demonstrated in a very low level through to the end of the experiment (>6 months PI) which can indicate that there was a poor involvement or low response of Th-1 in this infection (Fig 30-33).

3) The level of IgE-Ab showed significantly higher in the both infected group. In addition, blood eosinophilia could also demonstrated in human gnathostomiasis (Kraivichian et al., 2004) and infected animal (Saksirisampant, personal communication). The higher degree of infection (10 larvae) in those animal had higher percentage of blood eosinophil than the lower degree of infection (5 larvae) (Saksirisampant, personal communication).

4) The depressed lymphoproliferative response to mitogen and specific antigen stimulations (Table 8 and 9) suggested that the Th-1 cell response is defective.

The IgG1-Ab and IgE-Ab response in mouse gnathostomiasis of this study may applied for immunodiagnosis. Furthermore, the decrease of IgG1-Ab and IgE-Ab after antihelmintic treatment might be valuable for the evaluation of the effectiveness of chemotherapy.

In further studies, detailed mechanism of the T-cell subpopulation, which involves in the Th-2 response of this mouse-gnathostomiasis system, should be elucidated, particularly its cytokines production such as IL-2, IL-4 and IL-5. In addition, purification of the potential protein component of the advanced L3s somatic Ag is another important point to study. Because, highly complicated antigenic made up of this antigen had been demonstrated with more than 20 bands in SDS-PAGE (Priwan, 1985; Saksirisampant, 1986). By ELISA, cross-reaction of L3s Ag with other parasitic infections including angiostrongyliasis (Suntharasamai et al., 1985; Tuntipopipat et al., 1989), opisthorchiasis (Malinee, 1989), paragonimiasis, taeniasis and hook-worm infection (Welch et al., 1980; Tapchaisri et al., 1991; Saksirisampant et al., 2001) was also demonstrated. There was only few bands showed specific reaction with serum antibody in Western blot. The protein band of 24 kDa showed specific reaction with IgG-Ab, while bands of 43 and 49 kDa showed specific with IgE-Ab (Nopparatana et al, 1991; Tapchaisri et al., 1991; Saksirisampant, personal communication). Specific protein band (S) of IgG1-Ab and IgE-Ab, in acute and chronic infected sera and those in post-anthelmintic treated sera should be another further study as well. Furthermore, the practical preparation of acceptably pure antigens in gnathostomiasis serology may be likely the excretory-secretory antigens. Because, the nematode-secreted products are more closely related to active infection than somatic antibody response (Saksirisampant et al., 2001).

2. Lymphoproliferative responses of mice infected with *G.spinigerum*

The degree of depressed lymphoproliferative response to the Con A stimulation varied with both the intensity and the duration of infection. These decresed response to Con A stimulation was obvious in animals with high dose of *G.spinigerum* and in those with longer duration of infection (Day 180 PI).

With regard to antigen stimulation as expected, positive lymphoproliferation could be demonstrated in the infected group when compared to the normal control mice. However, a trend of decreased response to antigen stimulation was observed in the chronic infected group (Day 180 PI, both 10 and 20 larvae) compared to other groups, although it was not statistic significant.

These decreased responses were not due to aging of the animals because the data were comparable to the same age group control animals. In fact, the result presented in Table 2 and 3 showed that the lymphoproliferative responses of uninfected normal splenic lymphocytes taken from young (1-3 months) and old mouse (7-8 months) were not different. Thus, these data suggest that the immunodepression of infected mice is associated with the chronicity and heavy infection of *G.spinigerum*.

Immunosuppressions can be demonstrated in a number of parasitic infections eg. *Trichinella spiralis* (Faubert, 1982; Barriga, 1978), *Onchocerca volvulus* (Soboslay et al., 1999), *Taenia crassiceps* (Sciutto et al., 1995) and *Toxocara canis* (Allen et al., 1996) etc. These phenomenons were reversible. We wonder whether the reversible effect can be found in our *G.spinigerum*-mouse system. In this study, the animals were treated of infection by ivermectin. Interestingly, the lymphoproliferative responses to both Con A and antigen-stimulation were significant increased, particularly at experiment on Day 180 following treatment with ivermectin. The result of worm recovery from the same dose of ivermectin used in this study (0.4 mg/kg body weight) showed approximately 90% cure rate (Chawengkirttikul, personal communication).

In this experiment, the reversal of immunodepression to Con A and somatic antigen in *G.spinigerum*-infected mice was demonstrated after the parasites were removed. Although the direct effects of ivermectin on lymphocyte activity is not known. However, the residual effect of drug is highly unlikely; because the drug is rapidly metabolized in the liver. The apparent plasma half-life of ivermectin is approximately at least 16 hours following oral administration. Nevertheless, lymphoproliferative responses of the normal animals treated with ivermectin tested 6 months later were not altered when compared to the normal control mice (data not shown). Since, Con A stimulation index of ivermectin treated mice show the same order of magnitude as that of controls. In summary, the immunodepression in these infected animals was abolished by appropriated anthelmintic treatment, suggesting

that the immunodepressive effect can be reversible and is associated with active infection.

We hypothesize that the immunodepression observed with this infection is one mechanism that the parasite used to evade host defence. For this nematode, the infected mice are normally not able to eliminate the parasites and appear to have no resistance to reinfection. It was of interest to further characterize the mechanism of immunodepression in *G.spinigerum*. There are various possibilities as discussed below.

The depressed lymphoproliferative response to Con A and somatic antigen stimulation suggest that the T-cell response is defective. First, it is possible that the suppression may be due to a decrease in the proportion of responsive T cell, or to a reduced response of individual cells.

Second, it is possible that some antigen from the parasite may block directly at lymphocyte receptor. The excretory and secretory products of *Fasciola hepatica* had been reported to block rat lymphocytes. They might protect the parasite from it's host immune defenses (Mulcahy et al., 2004). However, there is no previous evidence that any of the *G.spinigerum* antigens is cytotoxic for the mice lymphocytes or accessory cells required for efficient immune response, which should be investigated in the future.

Third, it is possible that the suppression is due possibly to defects in interleukin-2 (IL-2) production. Decreased lymphocyte proliferation and IL-2 production have been reported in animals infected with various parasites, such as *Taenia crassiceps*, *Onchocerca volvulus*, *Toxoplasma gondii* and *Trypanosoma cruzi*. It was found that diminished IL-2 production during the infection with *Teania crassiceps*, might be a part for the diminished responses to Con A. The suppressed Con A response observed in this *Teania spp.* infection could be attributed at least in part to defects in IL-2 production. Since, the additions of recombinant IL-2 to cell cultures could restore responsiveness. Similar results were seen with the suppressive effect of *Onchocerca volvulus* secretion/excretion (S/E) antigens that can be overcome by addition of exogenous IL-2. Those results suggest that deficiency in IL-2 production rather than down-regulation of IL-2 receptor is responsible for inhibition of lymphocyte proliferative responses (Elkhalifa et al., 1991). However, the other explanations are the decreasing in affinity between the IL-2 receptor and

IL-2 in infected animal. Since, in the experiment of *Toxoplasma spp*. infection, the decreased lymphoproliferative responses of infected groups could be enhanced by exogenous IL-2 reconstitution, but their peak response never reached that of the control group. In addition, it has been reported that *Trypanosoma cruzi* inhibits the capacity of human T lymphocytes to express surface IL-2 receptor after lymphocyte activation which render T lymphocytes unable to receive the IL-2 signal (Beltz et al., 1988).

Fourth, it is possible that relationship of Th-2 responses and immune suppression of multicellular organisms might lead to profound down-regulation of host immune responses. Generation of suppressive cells which is dominated by a Th-2 type profile, are well-established features of human filarial infection. The induction of host IL-4 is characteristic of infection which is required for the generation of suppressive cells. Peripheral blood lymphocytes from people with active infection had altered proliferation to parasite antigen but with production of antigen-specific IL-4 (Allen et al., 1996).

In conclusion, the results indicate that mice experimentally infected with *G.spinigerum* had significant depression of the cellular response to Con A and somatic L3s antigen. The depression was more severe with prolonged and heavy infection. The immunodepression in these animals was abolished by anthelmintic treatment, suggesting that the immunodepressive effect is reversible and is associated with active infection. There are many possible mechanisms, which could explain immunodepression in the parasite which should be further studied. Exploration of the mechanism by which *G.spinigerum* induces depression of lymphocyte functions could help not only to further the knowledge of immune mechanisms that participate in this host-parasite relationship but also to advance understanding of the regulatory events governing lymphocyte activation.

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APPENDIX

1.2% Acid-pepsin

Pepsin A (Sigma, U.S.A.)	12 gm
Distilled water to	1 liter

Adjust pH to 2.0 with 1 N HCl

RPMI 1640 culture medium

RPMI 1640 (Gibco, U.S.A.)	10.4 gm
Penicillin-Streptomycin (Gibco, U.S.A.)	10.0 ml
1 M Hepes(Sigma, U.S.A.)	10.0 ml
Deionized distilled water to	1 liter

Adjust pH to 7.00 with 10% sterile $NaHCO_3$

Sterilization : By 0.22 μ millipore membrane filtration

1 M HEPES solution

Hepes (N-2-hydroxyethylpiperazine	23.82 gm
N-2-ethanesulfonic acid, Sigma)	
Distilled water to	100.0 ml

Sterilization : By 0.22 μ millipore membrane filtration

Scintillation fluid

PPO (2,5-diphenylosazole)	5.0 g
POPOP (1,4-bis-2-(5-Phenyloxazoly) benzene)	0.1 g
Toluene	1.0 liter

Coating buffer : 0.05 M carbonate pH 9.6

Na_2CO_3	1.59 gm
$NaHCO_3$	2.93 gm
Distilled water to	1 liter

Incubation buffer

Bovine serum albumin(Sigma, U.S.A.)	1 gm
Phosphate-buffered saline (PBS) pH 7.1	100 ml
Tween 20	0.05 ml

Washing buffer

NaCl	45 gm
Distilled water to	5 liter
Tween 20	2.5 ml

Blocking Solution

Bovine serum albumin(Sigma, U.S.A.)	0.1 gm
PBS pH 7.1	10.0 ml

Substrate solution

Orthophenylene diamine (OPD, Sigma, U.S.A.)	0.2 gm
Distilled water to	10.0 ml
35% H_2O_2	5 μl

Reaction stopping solution : 5N H_2SO_4

Conc.H_2SO_4	1.2 ml
Distilled water to	10.0 ml

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