IDENTIFICATION AND CHARACTERIZATION OF *GNATHOSTOMA* ANTIGENS WITH POTENTIAL FOR IMMUNODIAGNOSIS OF GNATHOSTOMIASIS

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ชื่อวิทยานิพนธ์ การศึกษาคุณสมบัติของแอนติเจนของพยาธิตัวจี๊ด *(Gnathostoma spinigerum)* ที่มีศักยภาพสำหรับพัฒนาเพื่อใช้ในการวินิจฉัยโรคพยาธิตัวจี๊ด (gnathostomiasis) โดยวิธีทางอิมมิวโนวิทยา

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บทคัดย่อ

โรคพยาธิตัวจี๊ด (gnathostomiasis) มีสาเหตุจากการติดเชื้อพยาธิตัวจี๊ด *(Gnathostoma spinigerum)* การติดเชื้อพยาธิตัวนี้มีรายงานมาจากหลายพื้นที่ในประเทศไทย การวินิจฉัยโรคในปัจจุบันนี้เป็นเพียงการสันนิษฐานจากอาการทางคลินิก เช่น อาการบวมเคลื่อนที่แบบเป็น ๆ หาย ๆ, มีอาการคันและปวดบริเวณที่บวมและบริโภคนิสัยของผู้ป่วย และจากผลการตรวจทางห้องปฏิบัติการบางอย่าง เช่น การพบเม็ดเลือดขาวชนิดอีโอซิโนฟิลสูงในในกระแสโลหิต (eosinophilia) การวินิจฉัยที่กล่าวมาข้างต้นนี้เป็นการวินิจฉัยที่ไม่แน่นอน ดังนั้น การวินิจฉัยโดยวิธีการทางอิมมิวโนวิทยาจึงน่าจะช่วยสนับสนุนการวินิจฉัยอาการทางคลินิกได้ อย่างไรก็ตาม จะต้องมีการพัฒนาการวินิจฉัยทางอิมมิวโนวิทยา เพื่อที่จะให้ได้วิธีการที่ไว (sensitivity) และจำเพาะแม่นยำ (specificity) ในการศึกษานี้ผู้วิจัยจึงพยายามวิเคราะห์คุณสมบัติของแอนติเจนที่เตรียมจากส่วนต่าง ๆ ของพยาธิ เช่น somatic extract, excretory-secretory products (ES) และ surface extract โดยวิธีทางสรีระเคมี (physicochemical technique) และ

อิมมิวโนวิทยา (immunological technique) เพื่อให้ได้องค์ประกอบที่มีศักยภาพสูงและมีความจำเพาะสูง สำหรับนำมาพัฒนาใช้ในการวินิจฉัยโรคได้อย่างแม่นยำ ในการศึกษาครั้งนี้ผู้วิจัยได้ทำการศึกษากลุ่มคนไข้ที่มีอาการทางสมองร่วมด้วย (cerebral gnathostomiasis) ซึ่งถือเป็นกลุ่มคนไข้ที่มีพยาธิสภาพที่รุนแรง และอาจทำให้ผู้ป่วยถึงแก่ชีวิตได้ โดยพยายามพัฒนาวิธีการที่เหมาะสมมากที่สุดสำหรับตรวจหาแอนติเจนของพยาธิในน้ำไขสันหลัง และหาแอนติบอดีทั้งในน้ำเหลืองและน้ำไขสันหลังของผู้ป่วย

ผลจากการศึกษาวิเคราะห์โดย SDS-PAGE พบว่า somatic แอนติเจนของพยาธิตัวจี๊ดมีองค์ประกอบค่อนข้างจะซับซ้อนประกอบด้วยสารจำพวกโปรตีนและไกลโคโปรตีนที่มีน้ำหนักโมเลกุลตั้งแต่มากกว่า 116 ลงมาถึง 13 กิโลดาลตัน ในขณะที่ ES และ surface extract ประกอบด้วยโปรตีนและไกลโคโปรตีนที่มีน้ำหนักโมเลกุล 98 ถึง 12 กิโลดาลตัน และ 70 ถึง 16 กิโลดาลตัน ตามลำดับ องค์ประกอบที่มีปริมาณมากที่สุดของ somatic แอนติเจนเป็นสารจำพวกไกลโคโปรตีนที่มีน้ำหนักโมเลกุล 38 กิโลดาลตัน ในขณะที่องค์ประกอบส่วนใหญ่ของ ES แอนติเจนซึ่งมีน้ำหนักโมเลกุลตั้งแต่ 55 ถึง 40 กิโลดาลตันเป็นสารจำพวกไกลโคโปรตีน และเมื่อทำการศึกษาวิเคราะห์โดยวิธี immunoenzymatic blotting พบว่าทั้ง somatic และ ES แอนติเจนสามารถทำปฏิกริยาที่จำเพาะกับน้ำเหลืองจากผู้ป่วย และสัตว์ที่ติดเชื้อพยาธิตัวจี๊ด นอกจากนี้ เรายังได้ทำการศึกษาความจำเพาะของแอนติเจนทั้งสองโดยให้แอนติเจนทั้งสองชนิดทำปฏิกริยากับน้ำเหลืองจากผู้ป่วยที่ติดเชื้อพยาธิหอยโข่ง *(Angiostrongylus cantonensis)* ซึ่งเป็นพยาธิตัวกลมที่สามารถทำให้เกิดพยาธิสภาพที่คล้ายคลึงกันในระบบประสาท จากการศึกษาดังกล่าวพบว่าองค์ประกอบที่มีน้ำหนักโมเลกุลน้อย (26, 21 และ 19 กิโลดาลตัน) ของ somatic แอนติเจนจากพยาธิตัวจี๊ด จะทำปฏิกริยาเฉพาะกับน้ำเหลืองจากผู้ป่วยที่ติดเชื้อพยาธิตัวจี๊ด และไม่ทำปฏิกริยากับน้ำเหลือจากผู้ป่วยที่ติดเชื้อพยาธิหอยโข่ง ในขณะที่องค์ประกอบที่มีน้ำหนักโมเลกุลมาก (มากกว่า 38 กิโลดาลตัน) จะทำปฏิกริยากับน้ำเหลืองที่ติดเชื้อพยาธิทั้งสองชนิด ในทางตรงกันข้าม ES แอนติเจนจะทำปฏิกริยาที่จำเพาะต่อน้ำเหลืองจากคนไข้ที่ติดเชื้อพยาธิตัวจี๊ด แต่ไม่ทำปฏิกริยากับน้ำเหลืองจากผู้ป่วยที่ติดเชื้อพยาธิหอยโข่ง

เพื่อที่จะให้ได้วิธีการวินิจฉัยโรคทางอิมมิวโนวิทยาที่มีความจำเพาะมากขึ้น ผู้วิจัยจึงได้พยายามพัฒนาวิธีการหาแอนติเจนของพยาธิตัวจี๊ดที่มีความไวและความจำเพาะสูงต่อ ES แอนติเจน โดยวิธี biotin-streptavidin ELISA (B-SA ELISA) ขึ้นมา แต่ถึงแม้ว่าวิธีการนี้จะสามารถวัดระดับความเข้มข้นของโปรตีนได้ต่ำถึง 2 ng/ml ก็ตาม ผู้วิจัยก็ยังไม่สามารถตรวจพบแอนติเจนในน้ำไขสันหลังของผู้ป่วยที่มีอาการทางประสาทร่วมด้วย จากจำนวนผู้ป่วย 28 ราย สามารถตรวจพบแอนติเจนได้เพียงรายเดียว ดังนั้นอาจเป็นไปได้ว่าในน้ำไขสันหลังของผู้ป่วยอาจจะมีแอนติบอดี้สูง ซึ่งทำให้การวัดหาแอนติเจนทำได้ไม่ดี ดังนั้น เราจึงพยายามวิเคราะห์ต่อว่าจะมีแอนติบอดี้ที่จำเพาะต่อ somatic แอนติเจนของพยาธิตัวจี๊ดในน้ำไขสันหลังของคนไข้หรือไม่ และหากมีระดับแตกต่างไปจากที่พบในน้ำเหลืองหรือไม่ จากการวิเคราะห์ครั้งนี้พบว่าทั้งน้ำเหลืองและน้ำไขสันหลังของผู้ป่วยกลุ่มนี้มีระดับของแอนติบอดี้ที่จำเพาะต่อ somatic แอนติเจนสูงกว่าในกลุ่มควบคุม และระดับที่พบในน้ำไขสันหลังในผู้ป่วยทุกรายจะต่ำกว่าระดับที่พบในน้ำเหลือง

เพื่อที่จะได้ทราบว่าแอนติบอดี้ในน้ำไขสันหลังของผู้ป่วยเป็นแอนติบอดี้ที่มาจากน้ำเหลืองหรือสร้างขึ้นโดยเซลล์ในระบบประสาทส่วนกลาง ผู้วิจัยจึงได้ทำการศึกษาหาความเข้มข้นของโปรตีนบางชนิดเพื่อนำมาคำนวนหาสัดส่วนของระดับอัลบูมินในน้ำเหลืองและน้ำไขสันหลัง (albumin ratio), สัดส่วนของระดับ IgG ในน้ำไขสันหลังและน้ำเหลือง หารด้วยสัดส่วนของระดับอัลบูมินในน้ำไขสันหลังและน้ำเหลือง (IgG-albumin index) และ specific antibody activity (specific IgG titer to somatic L3G/total IgG) ซึ่งจากการศึกษาดังกล่าวเราพบว่ามีคนไข้ 2 ราย จากจำนวนทั้งหมด 32 ราย สามารถตรวจพบว่ามีการสร้างแอนติบอดี้โดยเซลล์ในระบบประสาทส่วนกลาง และนอกจากตรวจหาแอนติเจนและแอนติบอดี้ในน้ำ ไขสันหลังของผู้ป่วยแล้ว การศึกษายังครอบคลุมไปถึงการตรวจหา immune complexes ในน้ำไขสันหลังด้วยวิธี complement-consumption test และพบว่ามีผู้ป่วย 1 ราย ที่สามารถตรวจพบ immune complexes ในน้ำไขสันหลังได้

ผลจากการศึกษาดังกล่าวทั้งหมดพอจะสรุปได้ว่าการตรวจหาแอนติเจนในน้ำไขสันหลังจากผู้ป่วยที่ติดเชื้อพยาธิตัวจี๊ดและมีอาการทางสมองร่วมด้วยไม่เหมาะสมสำหรับใช้ในการวินิจฉัยโรคพยาธิตัวจี๊ด ในขณะที่การตรวจหาระดับ

แอนติบอดี้ที่จำเพาะจะให้ผลการวินิจฉัยที่น่าเชื่อถือมากกว่า โดยเฉพาะอย่างยิ่งถ้าใช้องค์ประกอบที่ค่อนข้างจะบริสุทธิ์และทำปฏิกริยาเฉพาะกับน้ำเหลืองจากผู้ป่วยที่ติดเชื้อพยาธิตัวจี๊ด อย่างเช่น ES แอนติเจนซึ่งไม่ทำปฏิกริยากับน้ำเหลืองจากผู้ป่วยที่ติดเชื้อพยาธิหอยไข่ง นอกจากนี้ในผู้ป่วยกลุ่มดังกล่าวเราสามารถจะใช้น้ำไขสันหลังเป็นสิ่งส่งตรวจแทนน้ำเหลืองเพื่อตรวจหาระดับแอนติบอดี้ที่จำเพาะได้

Thesis Title: Identification and characterization of *Gnathostoma* antigens with potential for immunodiagnosis of gnathostomiasis

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ABSTRACT

Human gnathostomiasis caused by *Gnathostoma spinigerum* has been reported to be a prevalent nematode infection in many regions of Thailand. The diagnosis of this parasitic infection is only presumptive, based on clinical features, for instances, intermittent migratory swelling, itching, pain and history of consuming half-cooked meat by individuals in endemic areas. Attempts have been made to diagnose this infection by conventional laboratory method, such as, complete blood count which showed a high percentage of eosinophil in the circulation. A number of immunodiagnostic methods for gnathostomiasis have been developed to confirm the presumptive clinical diagnosis. However, the development of specific and sensitive immunodiagnostic method is essential to obtain a reliable diagnosis. In this study, the various third-stage larval *G. spinigerum* (L_3G) antigen preparations including somatic extract,

excretory-secretory product (ES) and surface extract were characterized, employing a number of physicochemical and immunological methods in order to define specific component(s) with potential for immunodiagnosis of gnathostomiasis. An additional study was also undertaken to detect these parasitic antigens in cerebrospinal fluid (CSF) of patients with central nervous system (CNS) involvements in order to monitor the active infection. Detection of specific antibodies in both serum and CSF of these cerebral gnathostomiasis patients had been undertaken in this study with a main objective of looking for the possibility of using CSF as a specimen for immunodiagnosis.

The SDS-PAGE pattern of somatic antigen was highly complex, consisting of proteins and glycoprotein with a molecular weight ranging from more than 116 to 13 KD. On the other hand, the ES antigen and surface extract consisted of components with more narrow molecular weight range of 98 to 12 KD and 70 to 16 KD respectively. The predominant somatic counterpart with a molecular weight of 38 KD was the only major glycoprotein detected in the somatic extract as demonstrated by concanavalin-A. On the contrary, a majority of the ES antigen, particularly those with molecular weight range of 55 to 40 KD, were glycoproteins. However, both somatic and ES antigens showed strong reactions with sera from infected humans, mice and rabbits immunized with various L_3G antigen preparations as demonstrated by immunoblotting technique. Specificity of these two antigens were analyzed with angiostrongyliasis serum obtained from patients suspected of having been infected with *Angiostrongylus cantonensis*, a common nematode found within CNS. The immunoblot

pattern showed that the low molecular weight components of the somatic antigen (26, 21 and 19 KD) reacted specifically with gnathostomiasis sera. On the other hand, those with high molecular weight components (more the 38 KD) reacted strongly with the angiostrongyliasis serum. On the contrary, the ES antigen failed to react with angiostrongyliasis serum.

In this study, the sensitive and specific biotin-streptavidin ELISA (B-SA ELISA) was also undertaken to be used for antigen detection in CSF of this group of patients. Although this B-SA ELISA could detect the presence of antigen to be level of 2 ng protein/ml, only one of the twenty-eight patients showed a positive antigen in his CSF specimen. It should be noted that no antibody could detect in his CSF. On the other hand, the other CSF specimens had high antibody levels in their CSF, therefore, any ES antigen may be in a form of immune complexes. In fact, one out of these CSF specimens, immune complexes were detected by complement consumption test.

An alternative approach with regarding to analyze both serum and CSF specimens of these patients for the presence of antibody reactive with the somatic antigen was also undertaken. Furthermore, by comparing the specific antibodies obtained in both serum and CSF of the individual patients and analyzed in conjunction with other immunological parameters, for examples, albumin ratio (serum albumin/CSF albumin), IgG-albumin index (CSF IgG/serum IgG ratio)/(CSF albumin/serum albumin ratio) and specific antibody activity (specific IgG titer/total IgG) all pointed to the fact that in addition to serum antibody which may be present in various degrees in CSF, local antibody production does occur within the CNS in two

cases out of thirty-two patients. An additional data clearly demonstrated that a large majority of the patients with CNS involvements gave high serum antibodies and the specific antibody could be readily detected in the CSF specimens.

From the overall study, it can conclude that the detection of antigen in CSF of cerebral gnathostomiasis patients is not suitable for immunodiagnosis. An alternative approach for detection of specific antibody is probably more effective, especially when the more refined ES component is available. The latter failed to react with angiostrongyliasis serum in immunoblotting analysis and therefore using the ES component as a diagnostic antigen would provide one with a reliable diagnosis for differentiation of gnathostomiasis and angiostrongyliasis. Either serum or CSF specimen can be used for antibody detection in cerebral gnathostomiasis patients.

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LIST OF ABBREVIATIONS

L_3G	*Gnathostoma spinigerum* third-stage larvae
ES	excretory-secretory antigen
CSF	cerebrospinal fluid
CNS	central nervous system
1% PBST	1% Tween 20 in phosphate buffered saline
BSA	bovine serum albumin
NSST	0.05% Tween 20 in normal saline solution
CNS-G	cerebral gnathostomiasis
SAH	subarachnoidal hemorrhage
EM	eosinophilic meningitis
UM	unidentified meningitis
B-SA ELISA	biotin-streptavidin enzyme-linked immunosorbent assay
L_3A	*Angiostrongylus cantonensis* third-stage larvae
CFD	complement fixing diluent
NHS	normal human serum
GVB	gelatin-veronal buffer

CHAPTER I

INTRODUCTION

It is well documented that a large number of zoonotic helminths, including trematodes, cestodes and nematodes can cause important and fatal diseases in man (1). Among infections caused by tissue nematodes, gnathostomiasis and angiostrongyliasis are considered to be important parasitic diseases found in many Asian Pacific countries, for instances, Thailand, Japan, Australia and Pacific islands (2-4). Human gnathostomiasis caused by *Gnathostoma spinigerum*, a nematode of cats and dogs (5), has been reported to be prevalent in certain areas of Central Thailand and is one of the public health problems in that region. A recent survey for gnathostomiasis throughout the country showed that there were about 900 suspected cases admitted to 92 provincial and Bangkok metropolitan hospitals (6). Humans acquire the infection by consumption of raw or undercooked fresh water fishes, eels, frogs and snakes harbouring encapsulated infective third-stage larvae. Although in the recent past, people have received better health education, the disease still persists, possibly because of their social customs and dietary habits.

CHAPTER II

BACKGROUND

1. Biology of *Gnathostoma spinigerum (G. spinigerum)*

G. spinigerum is a nematode whose life cycle involves mammalian definitive host and 2 invertebrate intermediate hosts. Adult *G. spinigerum* was first identified from gastric tumors found in a young tiger in London Zoo by Richard Owen in 1838. This species of *Gnathostoma* is now the most important member of the genus causing human gnathostomiasis in Thailand where at least three other distinct species of *Gnathostoma* have also been reported in various vertebrate definitive hosts; *G. hispidum* in the stomach wall of pigs, *G. doloresi* in the stomach wall of wild boars and pigs, and *G. vietnamicum* in the urinary system of otters (5).

1.1 Life cycle of *G. spinigerum*

In 1933 Prommas and Daengsvang first described a complete life cycle of *G. spinigerum* which is quite complex, requiring cyclops and fishes as first and second intermediate hosts respectively, and many carnivores, (for instances, cats, dogs and tigers) serve as definitive hosts (2, 3). Adult worms normally reside in the stomach wall of these mammals, inducing host reactions which may participate in development of a tumor mass. Adult females lay eggs which are periodically released through the openning of the tumor into the lumen of the stomach and finally pass out in the feces. The embryonated eggs hatch in fresh water and shortly develop into first-stage larvae. These larvae develop into second stage in the cyclops. In order to mature to infective third-stage

larvae, the second-stage larvae require second intermediate host, for example, fresh water fishes (2). Two species of fresh water fishes, namely cat-fish (*Clarias batrachus*) and snake-headed fish (*Ophicephalus striatus*), are the most common second intermediate hosts infected with this parasite. When definitive hosts consume these intermediate hosts, the infective third-stage larvae penetrate the stomach wall, enter the liver and muscle, while growing gradually in size. During migration in the tissue and before differentiating into adult stage, these larvae must undergo final molting. Subsequently, these immature worms re-enter the gastric wall and finally develop into sexually mature adults. The total time requires for completion of the life cycle in cats is about 7 months (2). There are at least 44 species of vertebrates reported to be susceptible to infection by *G. spinigerum* advanced third-stage larvae. These vertebrates include amphibians, reptiles, birds, and mammals. Fresh water fish is probably the most important intermediate host transmitting the infection to humans (2). In the humans, infection occurs by consuming raw or under-cooked fresh water fishes harboring these infective-stage larvae. However, penetration of intact or damaged skin by *G. spinigerum* larvae has also been demonstrated in experimental infection of mice, rats and cats (7), and prenatal transmission is known to occur in humans. In a more recent report, a small mature male worm was removed from the abdominal skin of a 7-days old baby (6). In general, man serves as an accidental host for this parasite; the worms migrate throughout all parts of human bodies, and occasionally cause severe disorders which can be fatal, particularly in the case involving central nervous system.

1.2 General morphology

The morphology of advanced third stage larvae which are the infective stage of *G. spinigerum* can be divided into 3 different parts, namely lips, head-bulb (or cephalic bulb) and body. Each head bulb bears four transverse rows of well-developed single-pointed hooklets. The number of hooklets varies from one row to another. The morphology and the arrangement of hooklets are used for the identification of species (5). The anterior half of the body is armed with cuticular spines which become less prominent towards the posterior end (5). This stage of parasite (3.95 x 0.42 mm) is commonly used as an antigen for immunological investigation. In one series of study, a total of 23 specimens were obtained from patients attending various hospitals and were sent to the Faculty of Tropical Medicine for identification (6). Five advanced third-stage larvae were obtained from different parts of the bodies and the size varied from 2.2-3.5 mm to 0.4-0.63 mm. Twelve of the 14 immature worms (8 males and 6 females) found had 8 rows of cephalic-hooklets. The two remaining immature worms had 6 and 7 rows, respectively. Immature male worms (4.6-9.3 mm to 0.6-1.05 mm) were slightly larger than immature female worms (3.8-16.2 mm to 0.83-1.0 mm). The remaining four cases were identified as mature males whose sizes varied from 9.9-12.5 mm to 1.0-1.25 mm.

2. Pathology of gnathostomiasis

Pathological changes induced by *G. spinigerum* are mainly due to *gnathostoma* toxins consisting of substances resembling acetyl-

choline, hyaluronidase, proteolytic enzyme and haemolysin (3). Mechanical action associated with migrating worm and host response may also contribute to these pathological changes. In humans, these pathological changes can be classified based on clinical manifestations into cutaneous, visceral and cerebral gnathostomiasis. Signs and symptoms of cutaneous gnathostomiasis on the skin or mucous membrane appear as intermittent migratory swellings of various circumscribed sizes lasting for one to two weeks. Some patients showed intense inflammatory reaction, itching or irritation with pain at the swelling areas. Visceral gnathostomiasis is usually associated with respiratory tract, gastrointestinal tract, genito-urinary tract and eyeball. However, cerebral gnathostomiasis is the most serious outcome of this nematode infection which may be fatal and is caused by the migration and presence of living worms in the brain tissue (8, 9). When cerebral gnathostomiasis occurs, the disease is known as eosinophilic myeloencephalitis; 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 (10). A severe agonizing pain over the trunk and lower extremities commonly appears before paralysis occurs (9). It was reported that infection by only one *G. spinigerum* could cause unconciousness and death when it involved CNS (2). The mortality of CNS gnathostomiasis was reported to be as high as 25 percent (11).

3. Immunological studies of *G. spinigerum* infection.

It has been demonstrated that both humoral and cell-mediated immune responses against somatic antigen of third-stage larvae (L_3G)

developed in animals experimentally infected with this parasite (12, 13). The immunological parameters used to detect these responses include the presence of haemagglutinating (12), precipitating and reaginic antibodies (14, 15). In experimentally infected mice, haemagglutinating (HA) antibodies reactive with advanced L_3G somatic extract could be detected as early as one week after infection (16). However, during this early stage of infection, the antibody titer was only 1:40 but the titer gradually increased to 1:1,280 eight weeks after the challenge. In another serological study on experimental gnathostomiasis, one group of mice were orally infected with 30 early L_3G (obtained from infected cyclops) while the other was infected with 3 advanced L_3G (obtained from infected mice) and the antibody responses were detected by Ouchterlony gel diffusion test (17). The group receiving 30 early L_3G showed precipitin bands at the 3rd week of infection but started to decrease at the 8th week and eventually became undetectable at the 11th week. The group receiving 3 advanced L_3G gave seropositive result as early as the 2nd week of infection and began to decline during the 4th week and became completely negative at the 6th week. These results showed that the initial antibody response depended on the stage, and not the number of the worms while the duration of the antibody response depended on the number of infected worms, and to a lesser extent on the stage of the worm itself.

In 1985, Priwan (16) tried to identify and to characterize the somatic protein components of advanced L_3G by SDS-PAGE. It was demonstrated that as many as 20 protein components with relative molecular weight ranging from 10 to 99 KD could be detected in the somatic extract and the predominant component had MW of 39 KD.

Further investigation by radioimmunoprecipitation and autoradiography suggested that a component with molecular weight of approximately 50 KD reacted specifically with sera obtained from mice habouring advanced L_3G.

Many groups of investigators attempted to study the immunological responses of patients with gnathostomiasis, especially with regard to immunodiagnosis. In 1985, Suntharasamai *et al.*,(18) studied IgG specific to third-stage larvae of *G. spinigerum* by ELISA. The results showed that using a cut-off titer of 1:400, sera from all parasitologically confirmed patients and patients with eosinophilic meningo-myeloencephalitis were positive, thus giving the test 100% sensitivity. On the other hand, only 56% of the sera from patients with cutaneous migratory swelling (CMS) were positive by the same method. The specificity of ELISA at the cut-off titer of 1:400 was 84% when compared with healthy blood donors, sera from patients with eosinophilic meningo-encephalitis due to *A. cantonensis* and sera from patients with other parasitic infections.

A more sensitive ELISA for the detection of IgG antibody to L_3G in the sera of patients with intermittent cutaneous migratory swellings was also reported by Dharmkrong-at and her colleagues (19) in 1986. In this study, a crude somatic extract of advanced L_3G obtained from naturally infected eels were used as an antigen. From a preliminary study, a single serum dilution at 1:320 was found to give satisfactory result. With this condition all sera with intermittent cutaneous migratory swelling were found to be positive when compared with those of normal healthy controls. However, sera from patients infected with *A. cantonensis* or with other intestinal round

worms also reacted weakly in the test system. This improved ELISA method was considerably more sensitive than the previous study which gave only 56% sensitivity in cutaneous migratory swelling group. The difference may be attributable to a difference in the sources of somatic antigens used. In the previous report, L_3G was obtained from experimentally infected mice, whereas in the latter study, the larvae were obtained from naturally infected eels, which were one of the common sources of infected meat consumed by humans. It is possible that the somatic components and the metabolic products of larvae obtained from different sources were antigenically distinct and detected different populations of antibodies.

One other interesting aspect of the humoral immune response in helminthic infection is an elevation of not only serum IgE antiparasitic antibody, but also of nonspecific immunoglobulin E level (20). It was suggested that any component of the nematodes could function as allergens which induced anaphylactic antibodies (21). However, the exact mechanism as to why allergenic molecules from nematodes induce a much stronger IgE antibody response than others is still unclear. During the course of helminthic infections, IgE antibody is enhanced, and one would expect that this isotype of antibody must be able to mediate a potent anti-helminthic effector mechanism, and this appears to be the case.

In 1987, Soesatyo *et al.*,(22) studied total and specific IgE to advanced L_3G from infected eels. The specific IgE responses in patients with parasitologically proven gnathostomiasis and with migratory swellings were markedly higher than in healthy controls. Similarly, total serum IgE in both groups of patients were also

elevated. However, there was only a weak correlation between specific IgE titers and total IgE levels. There was also weak correlation between specific IgG and IgE antibody titers in these patients.

4. *G. spinigerum* antigens and immunodiagnostic approaches

Gnathostoma is a metazoan parasite which has a wide variety of antigens capable of stimulating immune responses in mammalian hosts. In general, parasite antigens may be classified into three categories: surface or cuticular, excretory-secretory (ES), and somatic antigens. All developmental stages display these antigens. However, stage-specific antigens do occur, particularly in the ES antigens. Somatic antigens are commonly prepared in the form of aqueous extracts from appropriate developmental stages, whereas the ES products can be obtained from an *in vitro* maintenance or cultivation of living parasites in protein-free culture medium. The surface or cuticular components can be prepared by solubilizing the parasites with either ionic or non-ionic detergents.

In the past the immunodiagnostic method for parasitic diseases was the classical immunofluorescence, particularly the indirect fluorescent antibody (IFA) method. This technique has been widely used in viral, bacterial, fungal, protozoal, and helminthic infections (23). Although IFA test will undoubtedly continue to be of great value in the future, more simple diagnostic tests with equally high sensitivity and specificity are now available, for instance, enzyme-linked immunosorbent assay (ELISA). Since ELISA was introduced by Engvall and Perlmann (24) and by Van Weeman and

Schuurs (25), wide application in the field of diagnostic medicine, for examples, endocrinology, haematology, pharmacology, immunopathology and microbiology have been reported. The advantage of ELISA is that while it is as sensitive as radioimmunoassay (RIA), it is more simple to handle as it does not involve radio-active materials and is more economical to run as the reagents are cheaper and have longer shelf-life. Particularly, in the case of parasitic infections which infect millions of people, these factors must be taken into consideration when assessing the usefulness of any serological test (26). ELISA has therefore been used widely for mass screening. Recent studies using the ELISA have shown that patients with gnathostomiasis had serum IgG as well as IgE responses against the crude somatic extract of L_3G (18, 19, 22). The application of biochemical methods to separate the constituent molecules has allowed a more rational approach to investigate and to characterize parasite antigens. The characterization of the antibody responses against the various L_3G somatic antigens by SDS-PAGE and Western blot analysis with sera from parasitologically confirmed gnathostomiasis patients was investigated (27). The SDS-PAGE analysis of somatic L_3G revealed a complex pattern comprising of more than 40 protein bands with relative molecular weight ranging from 13 KD to 150 KD. Among these, at least 20 components were antigenic in humans. However, sera from normal healthy individuals also reacted with a few components of somatic antigen. These findings suggested that the serum antibody response against L_3G as analysed with Western blotting may be useful in a specific or confirmed immunodiagnosis of human gnathostomiasis. However, cross-reactivity with other parasites has not yet been ruled out.

5. STATEMENT OF PROBLEM

Parasitic infection is still a major public health problem in many parts of the world, particularly, in the tropical and subtropical areas. One of the important parasitic diseases in man is helminthic infection. Fluke and nematode infections are among the major important tropical diseases in the world. For tissue nematode infection caused by *G. spinigerum*, one of the main problems is lack of a reliable diagnostic method. Attempts have been made to diagnose this infection by using conventional laboratory methods, such as, complete blood count and cerebrospinal fluid (CSF) examination. Parasitological diagnosis is rare since the parasite can be recovered from only a small percentage of patients by surgical removal or spontaneous emergence of the worms through the pathological sites. It shows a doubtful result in the case of central nervous system (CNS) involvement (28). The diagnosis of eosinophilic meningitis syndrome associated with *G. spinigerum* is only presumptive. It is generally based on clinical features of meningeal or cerebral symptoms with eosinophilic pleocytosis caused by this parasite (11, 29). A history of consuming known intermediate or paratenic hosts caught in the endemic areas is helpful but not confirmatory. A confirmed or parasitological diagnosis is rare and therefore, a number of immunodiagnostic methods for gnathostomiasis have been developed, for instance, skin test and precipitin test (30-33). However, the results have been unsatisfactory due to insensitivity and/or non-specificity. Moreover, false positive due to immunological cross-reaction with other helminths is rather common (14, 15). Recently, specific serum IgG and IgE to somatic

antigens can be detected by ELISA technique in the sera of suspected patients (18, 19, 22). However, the specificity of test is still low, particularly, in differentiating it from cases infected with *A. cantonensis.* Attempt has been made to identify and to characterize specific antigens in order to increase the specificity of the diagnosis, particularly for the detection of antigens in the CSF in those patients with CNS involvement.

The aims of this present study are to

1. characterize and identify various L_3G antigens with potential in immunodiagnosis.

2. detect L_3G antigens in CSF of patients with CNS involvement in order to monitor the activity or severity of the disease by ELISA.

3. detect specific antibodies to L_3G somatic antigen in both serum and CSF of individual patients with a main objective of looking for the possibility of using CSF as a specimen for diagnosis.

CHAPTER II

MATERIALS AND METHODS

1. Method of collecting *G. spinigerum* third-stage larvae (L_3G)

The livers of naturally infected eels (*Monopterus albus*) were digested with 1% acid pepsin solution as described by Priwan (16). In brief, the livers were separated from the intestines and gall bladders with small forceps and then washed with 0.85% NaCl. In a typical experiment, 100 gram of livers were chopped into small pieces and placed in an Erlenmyer flask containing 300 ml of 1% pepsin A (BDH, Poole, England) containing 7 ml of concentrated hydrochloric acid and were allowed to digest overnight at 37°C. The digested livers were then filtered through a 500 µm stainless sieve and washed with tap water. Materials retained on the seive were transferred to a petridish and third-stage larvae were individually picked under a dissecting microscope. These larvae were suspended in Hank's Balanced Salt Solution (HBSS) (see Appendix) containing 200 units/ml of penicillin G (Sodium penicillin G, E. Merck, Darnstadt, Germany), 200 µg/ml of streptomycin (Streptomycin sulfate B.P., Glaxo Vidhyasom Ltd., Thailand) and 2 µg/ml of fungizone (Gibco, NY, U.S.A.).

2. Preparation of *G. Spinigerum* antigens

2.1 Third-stage larval somatic extract

Third-stage larvae suspended in a small volume of normal saline solution (NSS) were homogenized in a glass tissue grinder in the presence of 0.1 mM phenylmethylsulfonylfluoride (PMSF) and 0.1 mM L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK) (Sigma

St. Louis, Mo., U.S.A.). The homogenate was then sonicated in an ice-bath by an ultrasonic disintegrator (Soniprep 150, MSE Scientific Inc., Sussex, England) set to operate at 1 min intervals until most cells were broken as observed under a light microscope. The sonicated worms were kept overnight at 4°C to allow protein components to be more completely solubilized before being centrifuged at 10,000 rpm at 4°C for 30 min (SM 24 rotor, RC-5B Sorvall refrigerated centrifuge, Dupont Company, Newton, Con., U.S.A.). The supernatant fluid, referred to as crude somatic extract, was kept frozen in small aliquots at -20°C until used.

2.2 Third-stage larvae excretory-secretory antigen (ES)

2.2.1 The kinetics of excretory-secretory production

Third-stage larvae were washed 3 times, at 10 min interval, in HBSS containing antibiotics as indicated above. All reagents and media were sterile and all procedures were carried out with sterile technique in a laminar airflow (Environmental Air Control Incorporated, Hagerstown, Maryland, U.S.A.). After washing, the worms were cultured in Earle's basal medium (BME) (Gibco, Grand Island, NY, U.S.A.)(0.01 ml/worm) containing the same concentra-tion of antibiotics as used in the washing solution. The culture was carried out in a 10-ml Erlenmyer flask loosely plugged with cotton and was incubated at 37°C in a 5% CO_2 atmosphere (CH/P^{TM} CO_2 controller, Forma Scientific Inc., Ohio, U.S.A.).

In a typical experiment, a flask containing 1 ml of the culture medium was used for the culture of 100 third-stage larvae. The worms were observed for viability by their body contraction and motility. They were considered to be "dead" when they

were found to be non-motile, having pale color and elongated shape. By these criteria, dead worms could be readily distinguished from living worms and were immediately removed. The protein content of metabolic product (ES) was monitored by measuring OD_{280} of the spent medium at one-day interval for 14 days (Figure 1). Before measuring, the spent medium was centrifuged for 15 min at 2,500 rpm to pellet insoluble debris and the optical density of the supernatant fluid was measured at 280 nm using fresh BME as blank.

2.2.2 Bulk preparation of ES antigen

Worms were cultured continuously in the above condition for 4 days, the supernatant fluid was concentrated by ultrafiltration (Diaflow membrane, PM-10, Amicon Corp., Lexington, MA, U.S.A.), dialyzed overnight against NSS and was kept in small aliquots at -20°C until used.

2.3 Third-stage larval surface extract

The technique for solubilization of surface antigens using deoxycholate (DOC) was modified from the one described by Sutanto *et al.*, (34) for *B. pahangi*. Approximately 200 cultured worms were washed 3 times in an extracting buffer containing 10 mM Tris buffer pH 8.3, 2.5 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM PMSF and 0.1 mM TPCK. After washing, the worms were suspended in 1 ml of ice-cold 1% deoxycholate (DOC) (Merck, Germany) made in the above buffer system and the worm suspension was kept on ice for 3 hr with gentle shaking. The worms were subsequently removed by centrifugation at 2,000 rpm 10 min and the supernatant was dialyzed exhaustively against NSS and kept in small aliquots at -20°C until used.

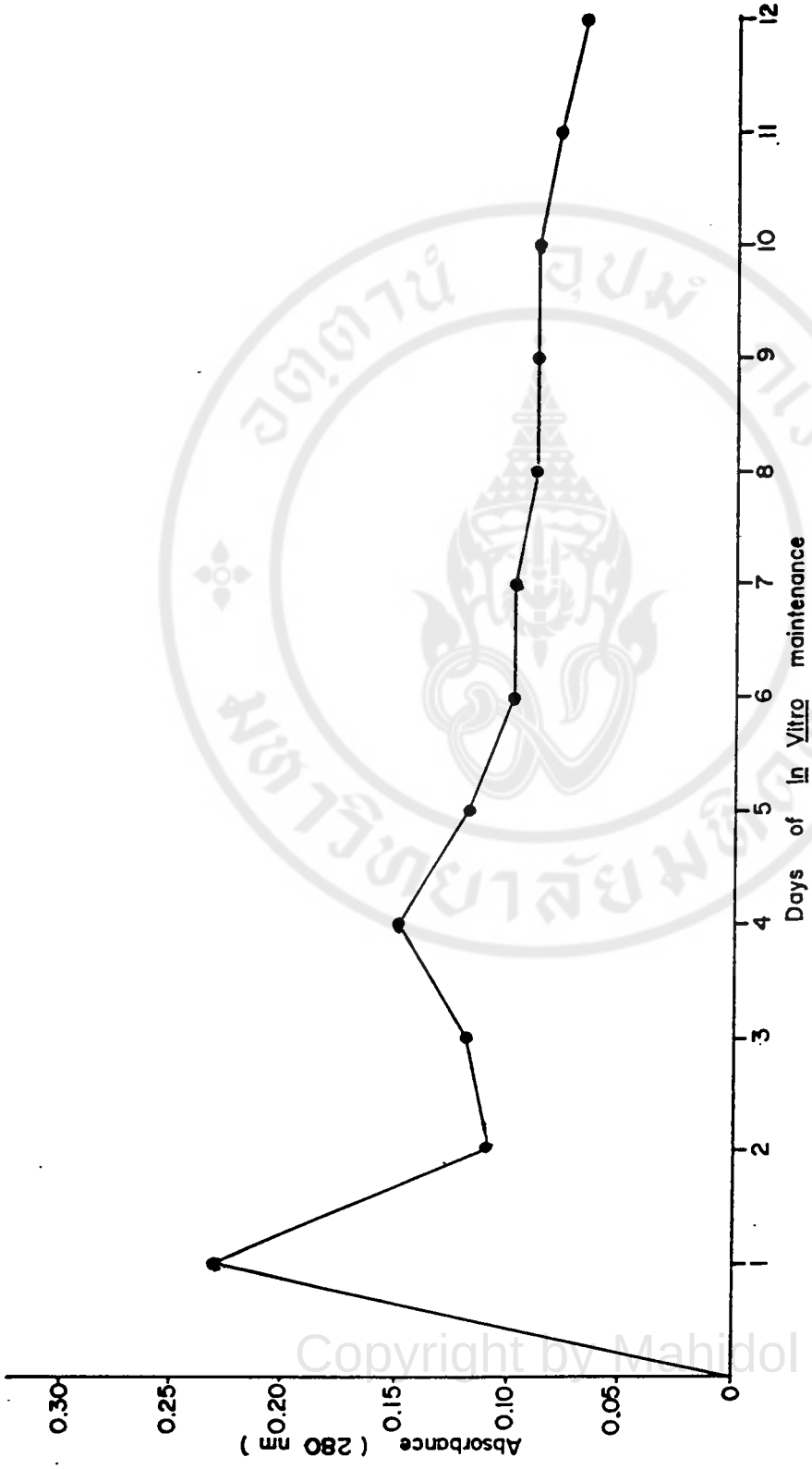


Figure 1 Metabolic activity of *G. spinigerum* third-stage larvae. The worms were maintained *in vitro* in BME medium and incubated at 37°C in 5% CO_2 atmosphere. The medium was changed every 24 hours; the spent medium was centrifuged and its protein content was expressed as optical density at 280 nm.

3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS) (35) was used to analyze the complexity of the protein components of the various *G. spinigerum* antigens and to determine the molecular weights of individual components as described by Weber and Obsborn (36).

3.1 Reagents and buffers

a) Polyacrylamide solution: A 30% (w/v) acrylamide (Sigma) stock solution and 0.8% (w/v) of N, N'-methylene-bis-acrylamide (Sigma) was prepared in distilled water by allowing it to dissolve at room temperature for 2 hr. Then the solution was filtered through a Whatman No. 1 filter paper before used.

b) Sodium dodecyl sulfate (SDS, Sigma) was prepared as a 10% (w/v) solution in distilled water.

c) Gel buffer for preparing the running gel was 0.375 M Tris-HCl pH 8.9.

d) Gel buffer for preparing stacking gel was 0.125 M Tris-HCl pH 6.8.

e) N,N,N',-N'-tetramethyethylenediamine (TEMED, Eastman Kodak).

f) Ammonium persulfate (May and Baker, Dagenham, England) was prepared as a 10% (w/v) solution in distilled water and used within 1 week.

g) Electrophoresis buffer (pH 8.3) was composed of 0.025 M Trizma-base, 0.192 M glycine and 0.1% SDS.

3.2 Preparation of slab gel

A glass block (9.6 cm x 12.7 cm x 1 mm) was set up by joining two pieces of glass plate (9.6 cm x 12.7 cm) with 1 mm thick spacers. The block was sealed with 1.5% agar to prevent leaking of the gel. Eight ml of a 10% acrylamide solution in 0.345 M gel buffer pH 8.9 containing 1% SDS, 0.05% (v/v) TEMED and 0.5% ammonium persulfate (running gel) was carefully added and allowed to polymerize at room temperature for at least 3 hr. The gel prepared as described above could be kept in a refrigerator if electrophoresis was not performed immediately. In either situation, 3 ml of a 4% stacking gel was layered over the running gel 1 hr prior to starting the current. The comb was gently inserted on the top of the gel to make slots for sample application.

3.3 Preparation of samples

Both samples and standard proteins were made up in sample buffer (see Appendix) containing 0.0625 M Tris-HCl pH 6.8, 1% SDS, 10% glycerol, 5% 2-mercaptoethanol (2-ME) and bromphenol blue and then heated 2.5 min in boiling water.

3.4 Electrophoresis

The electrophoretic chamber was prepared with cathode at the top and anode at the bottom. Electrophoresis was carried out with a constant current of 5 mA (500 Volts) per gel in a descending direction until the blue dye marker reached the bottom of the gel. Under this condition, it usually took about 5 hr to complete the running. Immediately thereafter, the exact distance of the dye migration and the gel length were measured.

3.5 Staining

3.5.1 Coomassie Brilliant Blue R. staining

Protein components in the gel slab were stained by soaking the gel overnight in a 0.2% Coomassie Brilliant Blue R (Sigma) solution containing 7% acetic acid and 5% methanol. Non-specific staining was removed by placing the gel in destaining solution (see Appendix) until the background stain was clear and specific protein bands could be easily visualized. After destaining, the length of the gel was recorded.

3.5.2 Silver staining

Silver staining was occasionally used to stain minor protein bands, because it is 100 times more sensitive than the Coomassie blue staining (37). The technique could detect as little as 0.01 nanogram of protein per square millimeter. Moreover, gels previously stained with the Coomassie blue could be restained by the silver.

In brief, the gel was fixed in a solution of 12% acetic acid in 50% methanol for 20 min. Sodium lauryl sulfate was removed from the gel by immersing it in a 10% ethanol in 5% acetic acid solution for 10 min. It was then soaked for 5 min in 0.0034 M potassium dichromate in 0.0032 N nitric acid and washed 4 times in deionized distilled water prior to being transferred to a 0.012 M silver nitrate solution. After 30 min, the gel was rinsed twice with 0.28 M sodium carbonate solution containing 0.05% formalin and allowed to soak with agitation in the same solution until protein bands appeared. When the staining pattern reached a desired intensity, the reaction was stopped by immediately soaking the gel

in a 1% acetic acid for 5 min and then washed thoroughly with distilled water. The stained gel could be kept in water for several months for further observation.

3.6 Molecular weight determination

A procedure used for molecular weight determination was modified from the method described by Weber and Obsborn (36) and by Davies and Stark (38). The molecular weight could be determined by comparing the electrophoretic mobility of the component to be determined with standard protein markers. A linear relationship was obtained when the relative mobility of standard markers were plotted against the logarithmic values of their molecular weight (Figure 2). The standard proteins used in the present study were β-galactosidase (116 KD; Sigma), Phosphorylase b (97.4 KD) bovine albumin (66 Kd), egg albumin (45 KD), trypsinogen (24 KD), β-lactoglobulin (18 Kd) and lysozyme (14.3 KD). The relative mobility of any unknown component was calculated from the following formular.

$$\text{Relative mobility} = \frac{\text{Distance of protein migration}}{\text{Gel length after destaining}} \times \frac{\text{Gel length before staining}}{\text{Distance of dye migration}}$$

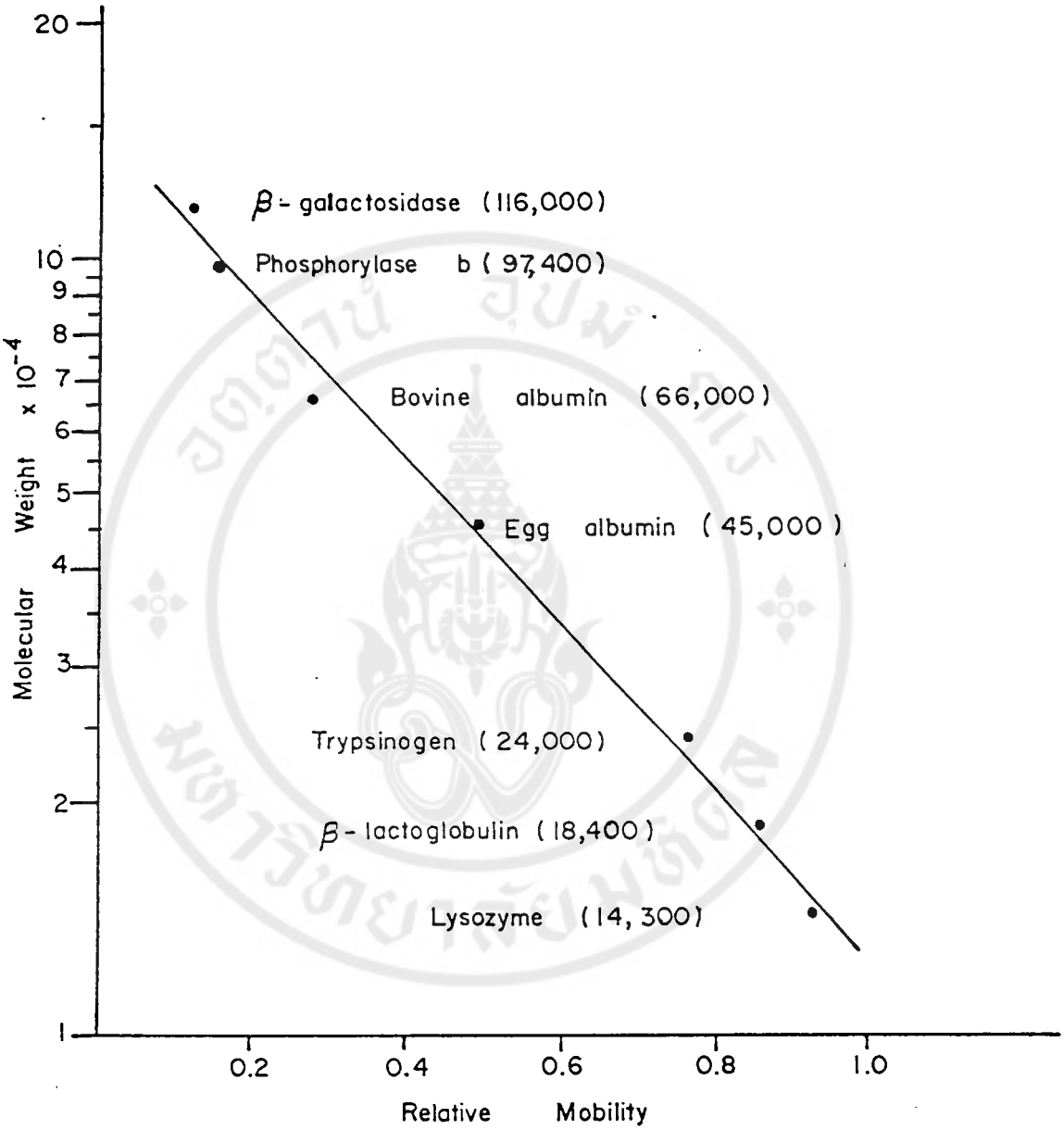


Figure 2 A typical standard curve used for molecular weight estimation of various L_3G antigen preparations by SDS-PAGE.

4. Immunoblotting and immunoenzymatic staining

Immunoenzymatic blotting was used to analyze and to characterize the complexity and antibody responses against the various components of L_3G.

4.1 Reagents and buffers

a) Towbin's buffer was made from 25 mM Tris-HCl, 192 mM glycine and 20% (v/v) methanol.

b) Phosphate buffered saline (PBS) pH 7.1 consisting of 0.85% NaCl, 7.5 mM Na_2HPO_4, 2.5 mM $NaH_2PO_4.2H_2O$.

c) PBST was a PBS containing 1% v/v Tween 20

d) Substrate buffer consisted of 18 mM Na_2CO_3, 32 mM $NaHCO_3$ and 0.5 mM $MgCl_2$

e) Substrate was prepared by mixing 120 mg o-dianisidine tetrazotized (Sigma) in 10 ml of substrate buffer with 10 mg β-naphthyl phosphate (Sigma) in 10 ml substrate buffer and filtered through a Whatman No. 1 filter paper. It was used immediately after mixing.

f) Substrate solution for horseradish peroxidase was prepared by mixing a 3, 3'-diaminobenzidine (Sigma) 0.3 mg/ml in 50 mM Tris-HCl pH 7.4 with H_2O_2 at a final concentration of 0.05%.

4.2 Electrophoretic blotting procedures

Proteins were first electrophoresed by SDS-PAGE (See Section 3). The size of gel used in the immunoblotting was 16 cm x 18 cm x 1.5 mm and was made from a 10% acrylamide solution in 0.375 M Tris-HCl pH 8.8 containing 1% SDS, 0.05% (v/v) TEMED and 0.5% ammonium persulfate (running gel), and 3.5% acrylamide solution in

0.125 M Tris-HCl pH 6.8 as stacking gel. The electrophoresis was performed with a constant current of 30 mA/gel at 15°C in an LKB 2001 Vertical Electrophoresis system. Each slot contained specimen with approximately 50 µg protein in a total volume of not more than 200 µl.

For Western blot analysis, the SDS-PAGE resolved components were electrophoretically transferred to a nitrocellulose filter membrane (pore size 0.45 µm, Millipore) in an LKB 2005 Transphor Electroblotting unit, using 0.6 ampere for an overnight transfer at 15°C, using Towbin's buffer as electrode buffer (39).

4.3 Staining for proteins

The proteins on the nitrocellulose membrane were stained with a 0.1% Amido black made up in 45% methanol and 10% acetic acid) and destained with 45% methanol containing 10% acetic acid.

4.4 Staining for glycoproteins

The electrophoretic blots on the nitrocellulose were immersed into a large volume of PBST for 30 min at 37°C. They were soaked with continuous shaking in 3% BSA in PBST for 2 hr at room temperature to block residual binding site on the membrane. Then, the membrane strips were washed 3 times with 10 mM Trizma base pH 8.0 (3 min for each washing) and then allowed to react with concanavalin A (Sigma) at a concentration of 200 µg/ml in 10 mM Trizma base. The reaction was allowed to take place with continuous shaking overnight at 4°C. The strips were washed 5 times in 10 mM Trizma base pH 8.0 (20 min intervals) at room temperature, then incubated with 100 µg/ml horseradish peroxidase (Sigma) diluted with

3% BSA in 1% PBST for 2 hr at room temperature. The strips were washed again as described above before a substrate solution was added and the reaction was allowed to take place until a desired color intensity developed, at which time the reaction was stopped by washing with distilled water.

4.5 Immunological detection of proteins on nitrocellulose

The electrophoretic blots were immersed into a large volume of PBST and kept at 37°C for 30 min. The membrane strips were soaked in 3% BSA in PBST for 2 hr with continuous shaking at room temperature and then washed 3 times in PBST (3 min each washing). The strips were allowed to react for 2 hr at room temperature with specific antibody appropriately diluted in 1% PBST and were then washed as described above. A predetermined dilution (in 3% BSA-PBST) of species-specific anti-immunoglobulin (goat anti-human IgG, goat anti-rabbit IgG (Sigma) or rabbit anti-mouse immunoglobulin (Dakopatts) conjugated with alkaline phosphatase was added and allowed to react for 1 hr at room temperature. The strips were again washed thoroughly as described before a substrate solution was added. The container was kept under gentle rotating motion until color developed and the enzymatic reaction was stopped by washing with tap water and dried on a glass plate.

5. Immunization procedure for preparation of antisera to *G. spinigerum* antigens

5.1 Anti-*G. spinigerum* somatic antigen

Rabbit antiserum to somatic antigens was prepared by immunizing the rabbit with 1 ml of a 1 mg/ml somatic extract mixed with

an equal volume of complete Freund's adjuvant. The rabbit was given the crude somatic antigen in the two front footpads and four other subcutaneous sites on the back. After a rest period of approximately one month, the rabbit was bled. Blood was allowed to clot, and the serum was seperated, dispensed into small aliquots and kept frozen at -20°C until used.

5.2 Anti-*G. spinigerum* ES antigen

Rabbit antiserum to ES antigens was prepared by immunizing the rabbit with 1 ml of a 0.25 mg/ml ES antigens mixed with an equal volume of complete Freund's adjuvant. The route of injections and bleeding period were the same as described for the somatic antigen. About six months after the first injection, the animal was boostered with 1 ml of a 0.25 mg/ml ES antigens in an equal volume of incomplete Freund's adjuvant at the same previous injection sites. Approximately one month later, the rabbit was boosted again with 1 ml of ES in aqueous solution (0.25 mg) by an intramuscular route and followed by an intravenous injection of the same amount of ES on the next day. The rabbit was bled 7 days after the last injection. Blood was allowed to clot and the serum was seperated and kept frozen at -20°C until used.

5.3 Anti-*G. spinigerum* surface antigen.

Rabbit antiserum to surface antigens was prepared by immunizing a rabbit with 0.5 ml of a 20 µg/ml surface antigens mixed with an equal volume of complete Freund's adjuvant. The route of injections were the same as described for the somatic extract. After a rest period of approximately one month, the rabbit was

boosted by a subcutaneous route with 0.5 ml of a 20 µg/ml surface antigens in an equal volume of incomplete Freund's adjuvant. Approximately one month later, the rabbit was boosted again with 0.5 ml (20 µg/ml) of surface antigen in aqueous solution by an intramuscular route. The animal was bled 7 days after the last injection.

6. Purification of rabbit immunoglobulin G (IgG)

6.1 Precipitation of rabbit globulins with ammonium sulfate

Rabbit hyperimmune serum was diluted with an equal volume of 0.85% NaCl. An equal volume of ice-cold saturated ammonium sulfate solution was added dropwise to the diluted serum with gentle stirring at 4°C. The mixture was kept stirring at this temperature overnight in order to get maximal precipitation. The precipitate collected after centrifugation at 10,000 rpm 4°C for 30 min (SM 24 rotor, RC-5B Sorvall refrigerated centrifuge, Dupont Company, Newton, Conn., U.S.A) was dissolved in 0.85% NaCl (NSS) to the original serum volume. Residual ammonium sulfate was removed by dialysis at 4°C against several changes of NSS until all ammonium sulfate was removed, as tested with 1% $BaCl_2$. The globulin fraction was then dialyzed against three changes of 0.02 M phosphate buffer pH 8.0, a starting buffer used for ion-exchange chromatography to be described in Section 6.2.

6.2 Purification of rabbit IgG by ion-exchange chromatography

Rabbit IgG was purified from the ammonium sulfate precipitated globulin fraction by an anion-exchange (DEAE cellulose) column chromatography. Approximately 1 gram of pre-swollen DEAE-cellulose

was used for the purification of IgG normally present in 1 ml of serum.

Precycling equilibration and packing of the DEAE cellulose (DE-52, Whatman Ltd., Springfield Mill, Maidstone, Kent, England) was done essentially as suggested by the manufacturer. In brief, the pre-swollen DEAE-cellulose was suspended in distilled water and fine particles were removed by slowly decanting the supernatant fluid after larger particles had settled. Then, the cellulose was equilibrated with 0.02 M phosphate buffer pH 8.0 (See Appendix). The globulin fraction obtained by ammonium sulfate precipitation and dialyzed against the DEAE equilibrating buffer was applied to the column (2.5 cm x 20 cm for 10 ml serum) and the protein was eluted with the same buffer at a rate of 0.5 ml/min. The profile of IgG eluted with this starting buffer is shown in Figure 3. Appropriate IgG fractions were collected and pooled. The protein content was estimated from the optical density (OD) value at 280 nm. The pooled IgG was dialyzed against 0.1 N NH_4HCO_3 pH 7.8, lyophilized and kept at 4°C.

7. Biotin conjugation of rabbit IgG

Rabbit IgG to *G. spinigerum* was covalently conjugated with biotin derivative by a method described by Nerurkar *et al.*, (40). Briefly, purified IgG at a concentration of 2 mg/ml was dialyzed overnight against 0.1 M $NaHCO_3$ pH 8.2 at 4°C. It was clarified by centrifugation at 1,000 rpm 4°C 10 min and the protein concentration was adjusted to 1 mg/ml with 0.1 M $NaHCO_3$ pH 8.2. A 1 mg/ml of N-

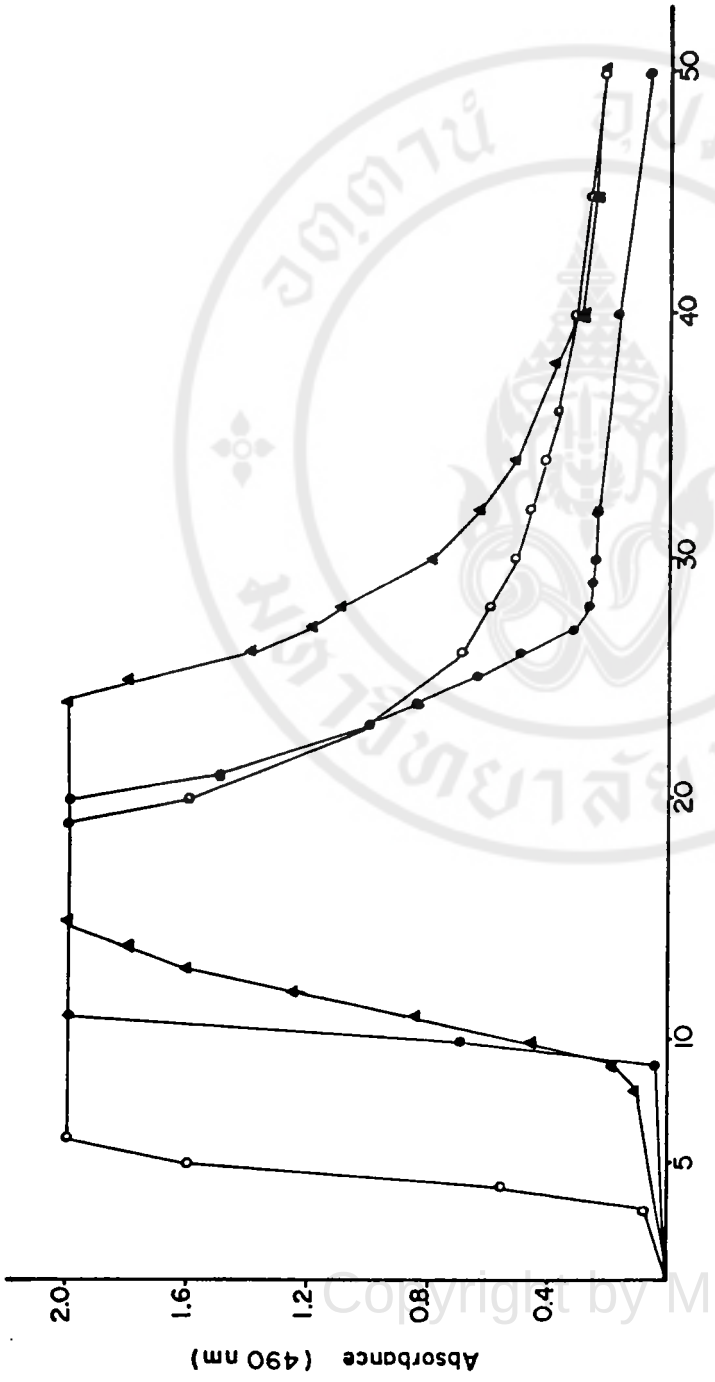


Figure 3 Fractionation of rabbit IgG containing antibody against somatic antigen (o-o-o), ES antigen (●-●-●) and surface antigen (▲-▲-▲) on a DEAE-cellulose column.

hydroxysuccinimidobiotin (Sigma) solution was freshly prepared in dimethylsulfoxide (DMSO) and then added immediately to the IgG solution. An optimal biotin/IgG ratio were predetermined by prior checkerboard titration and found to be 0.12/1, 0.17/1, 0.12/1 (mg/mg) for IgG anti-somatic, anti-ES and anti-surface antigens, respectively. The reaction mixture was allowed to take place at room temperature for 4 hr and were exhaustively dialyzed for 48 hr against 0.15 M PBS at 4°C to remove unconjugated biotin and DMSO. The supernatant was mixed with an equal volume of glycerol and stored at -20°C.

8. Sources of specimens

8.1 Experimentally infected animals

Sera from mice experimentally infected with advanced L_3G was provided from Mrs. Runglawan Chawengkirttikul (Department of Microbiology, Faculty of Science, Mahidol University). The animals were infected by oral feeding with L_3G and bled approximately 8 weeks after the initial infection.

8.2 Rabbit antisera to various L_3G antigens

Preimmune and immune sera to somatic extract, ES and surface antigens were prepared in rabbits as described in Section 5.

8.3 Human sera and cerebrospinal fluid (CSF)

8.3.1 Serum from patient with proven gnathostomiasis

Serum from a single patient with parasitologically proven gnathostomiasis was obtained from the Out-Patient Clinic of Siriraj Hospital. The worm was removed from the eye of the patient.

8.3.2 Positive reference sera

8.3.2.1 Positive gnathostomiasis sera

A number of individual sera from the Out-Patient Clinic of Ramathibodi and Chulalonkorn Hospitals were titrated for IgG antibody to L_3G somatic antigen by ELISA. Those with high titers were pooled, aliquoted and kept at -20°C until used.

8.3.2.2 Positive angiostrongyliasis sera

High titer sera from a number of patients infected with *Angiostrongylus cantonensis* were kindly provided by Dr. A. Dharmkrong-at, Department of Microbiology, Faculty of Science, Mahidol University.

8.3.3 Normal healthy control sera

Sera to be used as negative serum controls in this study were obtained from 22 apparently healthy adults who resided in non-endemic areas. Stool examination gave no evidence of any current parasitic infection. These sera were pooled and aliquoted as above.

8.3.4 Human cerebrospinal fluid (CSF)

A number of CSF specimens from patients with diverse signs and symptoms of CNS disorders, including gnathostomiasis, were sent from clinicians from many hospitals, for instances, Siriraj, Ramathibodi, Chulalongkorn and Khon Kaen hospitals. Only those CSF specimens available in quantity sufficient for detailed analysis were used for this study. Paired serum was available from a number of these patients. Some of these specimens were from patients with

known etiology; others were from those with signs and symptoms consistent with other CNS diseases (e.g., meningitis, myelitis, medulloblastoma and headache). Medical history including habits of consuming raw or poorly cooked foods was taken into consideration for analysis of *Gnathostoma* titers as they had signs and symptoms consistent with cerebral gnathostomiasis. The diagnostic slips from this group of patients were marked as eosinophilic myeloencephalitis (EM), subarachnoidal haemorrhage (SAH), cerebral gnathostomiasis (CNS-G) and some without any clinical remark.

For simplicity, the CSF used in this study were arbitrarily grouped into the following categories:

Group I: 5 patients with clinical manifestations suggestive for cerebral gnathostomiasis (CNS-G).

Group II: 2 patients with subarachnoidal haemorrhage (SAH).

Group III: 4 patients with eosinophilic meningitis, presumably due to gnathostomiasis (EM).

Group IV: 3 patients with unidentified meningitis (UM).

Group V: 14 patients with other CNS involvements.

Group VI: 4 patients with unidentified illnesses.

9. Enzyme-linked immunosorbent assay (ELISA) for detection of *G. spinigerum* antigens.

The antibody captured biotin-streptavidin enzyme-linked immunosorbent assay (B-SA ELISA) modified from that of Nerurkar *et al.* (41) was used to quantitate *G. spinigerum* antigens in clinical specimens. The assay was carried out in rigid, non-sterile, U-

bottom, 96-well polystyrene plates (Immulon II, Alexandria, Virginia, U.S.A.). Optimal conditions for each step were predetermined by appropriate checkerboard titration as described by Voller *et al.*, (42) as follows.

9.1 Optimization of the assay conditions

The optimal concentrations for IgG-anti *G. spinigerum* antigens (anti-somatic, anti-ES and anti-surface) and for the corresponding antigens used for the construction of standard curves were predetermined by checkerboard titration (42). The concentrations used are shown in Table 1. The reaction time between IgG-anti *G. spinigerum* and their respective antigens varied from 1 to 3 hr at 37°C, and under the condition used the most suitable time was found to be 1 hr.

Both the optimal reaction time and the optimal dilution of biotin-conjugated rabbit IgG were determined. The reaction times were found to vary from 30 to 120 min at 37°C with the biotin-conjugated rabbit IgG diluted between 1:100 to 1:800.

The optimal conditions of streptavidin conjugated peroxidase were similarly determined. The reaction times were found to vary from 20 to 60 min at 37°C with the streptavidin-conjugated peroxidase diluted between 1:500 to 1:2,000 for somatic and surface system, and 1:1,000-1:4,000 for ES system.

The enzymatic reaction was allowed to take place in the dark at room temperature and, depending on the system used, the time of reaction varied from 30 to 60 min.

Reproducibility of the ELISA used for antigen detection was evaluated by establishing a standard curve for the 3 *G. spinigerum*

Table 1 Protocol for a checkerboard titration of rabbit IgG anti-various L_3G antigens used for the quantitation of specific antigens by ELISA.

Antigen to be measured	Antigen concentrations for standard curve (ng/ml)	IgG antibody concentrations (µg/ml)
Somatic	4.0 - 1,000	5 - 20
ES	2.0 - 500	5 - 20
*Surface	15.6 - 1,000*	10 - 40

Somatic = third-stage larval crude extract.

ES = excretory-secretory product.

Surface = deoxycholate surface extract.

*used third-stage somatic crude extract as a standard instead of surface extract which was available in limited quantity.

antigens and the procedure was repeated several times on different occasions. The inter-run coefficient of variation (% cv) of reference standards was calculated in order to estimate the reproducibility of the test.

Sensitivity of the test was estimated from the dose response curve of the reference standards.

Specificity of the test was evaluated by using protein antigens from other parasites, i.e., *O. viverrini, S. mansoni, Taenia crassiceps, T. hydatigena* (obtained from Marcia Phoads, Livestock & Poultry Sciences Inst. Washington D.C., U.S.A) and *A. cantonensis* third stage larvae and female adult crude extracts, instead of the homologous *G. spinigerum* antigens used for the construction of standard curves. Other conditions were the same as indicated previously.

9.2 Determination of *G. spinigerum* antigens in cerebrospinal fluid

The IgG fractions of rabbit anti-*G. spinigerum* were diluted in 0.05 M carbonate buffer pH 9.6 to final protein concentration of 10 µg/ml, and 20 µg/ml and 20 µg/ml for somatic, ES and surface system, respectively. Each well of the U-bottom Immulon II plates was coated with 50 µl of the IgG solution at 4°C overnight. The plates were washed three times with 0.05% Tween 20 in NSS (NSST) for 3 min each. Unbinding sites were blocked with 2% BSA in PBS (See Appendix) at 37°C for 60 min. After washing as previously described, 50 µl of appropriate antigen (in 1% BSA in PBST) was added and standard curve for each antigen were

established. After incubation at 37°C for 1 hr, the plates were washed and 50 µl of a 1:200 dilution of the biotin-conjugated IgG fraction of the same rabbit antiserum was added and allowed to react at 37°C for 1 hr. After washing, 50 µl of a 250 µg/ml streptavidin-conjugated peroxidase (Sigma) at dilution at 1:1,000, 1:1,000, and 1:2,000 for somatic, ES and surface system, respectively, were added. After 1 hr of incubation at 37°C, the plates were washed and then 100 µl of freshly prepared substrate solution (See Appendix) was added. The enzymatic reaction was carried out at room temperature for 40 min and the reaction was stopped by the addition of 25 µl of 5 N H_2SO_4. The optical density was read at 490 nm (Minireader II Photometer, Dynatech Laboratories., Inc.). The antigens were considered to be present in significant quantity if the resulting OD of the specimen was > 2 times of the average OD of the reagent control.

Any specimen showing the presence of antigen by conventional B-SA ELISA was subsequently confirmed by a B-SA ELISA blocking test slightly modified from Yolken *et al.*, (43). The test was performed by first absorbing the specimens with excess preimmune and postimmune serum prior to being used in the conventional B-SA ELISA and a reduction of the OD obtained with unabsorbed specimens was determined. The percentage of blocking was calculated as

$$\frac{\text{O.D. (preimmune)} - \text{O.D. (postimmune)} \times 100}{\text{O.D. (preimmune)}}$$

O.D. (preimmune) and O.D. (postimmune) represented the optical density values obtained from the same unknown specimens after incubation with preimmune rabbit IgG and hyperimmune rabbit IgG

respectively. This test was considered to be positive if the percent blocking was at least 50%.

10. Quantitation of protein

10.1 Quantitation of total human IgG

Total IgG levels in both serum and CSF specimens were quantitated by ELISA (Bureerug T., personal communications). In brief, each well of U-bottom Immulon II micro ELISA plates was coated at 4°C overnight with 50 µl of a 1.25 µg/ml rabbit anti-human IgG (obtained from Bureerug, T.). The plates were washed as before and the unbinding sites were blocked with 100 µl of a 2% BSA in PBST at 30°C for 1 hr. Serum specimens to be quantitated were diluted 1:2,500, 1:12,500, 1:62,500 and 1:312,500 and CSF specimens were diluted 1:10, 1:50, 1:250 and 1:1,250. Pooled normal human serum with known IgG concentration was used to establish a standard curve. Fifty microliters of a 1:2,400 peroxidase-conjugated rabbit anti-human IgG (Dakopatts) was then added and allowed to react at 30°C for 1 hr. One hundred microliters of the substrate solution was added and the enzymatic reaction was allowed to proceed at room temperature for 40 min. The enzymatic reaction was stopped with 25 µl of 5N H_2SO_4. A typical standard curve is shown in Figure 4.

10.2 Quantitation of specific IgG to somatic extract of L_3G

The levels of specific IgG to somatic *G. spinigerum* in serum and CSF specimens from the patients were titrated by ELISA (data from Chawengkirttikul, R.). In general, wells of U-bottom Nunc-Immuno II (Inter Med Nunc, Denmark) was coated with 50 µl of a 5 µg/ml somatic extract from naturally infected eels at 4°C

overnight. The plates were washed as before and unbinding sites were blocked with 200 µl of 2% BSA in PBS at 30°C for 1 hr. Fifty microliters of a serial 5-fold dilution of serum and CSF were incubated at 30°C for 1 hr. After washing the plates as previously described, 50 µl of a 1:900 peroxidase-conjugated rabbit anti-human IgG were added and allowed to react at 30°C for 1 hr. The plates were washed as described above and the enzymatic activity was determined by the addition of 100 µl of substrate solution and the enzymatic reaction was allowed to proceed at room temperature for 40 min. The reaction was terminated by the addition of 25 µl of 5 N H_2SO_4.

10.3 Quantitation of albumin

Albumin levels in the serum and CSF specimens from the patients were quantitated by ELISA (data from Chawengkirttikul R.). In general, wells of U-bottom Nunc-Immuno II was coated with 50 µl of a 5 µg/ml IgG fraction of rabbit anti-human albumin at 4°C overnight. The plate was then washed with 1% gelatin in 0.05% Tween 20 NSS (NSST) as indicated previously. Unbinding sites were blocked with 1% gelatin in NSST at 30°C for 1 hr. Sera were diluted 1:100,000, 1:1,000,000, 1:2,000,000 and 1:4,000,000 and CSF specimens were diluted 1:5,000, 1:10,000 and 1:20,000 with 1% gelatin in NSST. Crystalline human serum albumin was used for the calibration of standard curve which was established for each plates. Fifty microliters of a 1:5,000 dilution of peroxidase-conjugated rabbit anti-human albumin was then added and allowed to react at 30°C for 1 hr. The enzymatic activity was determined as described

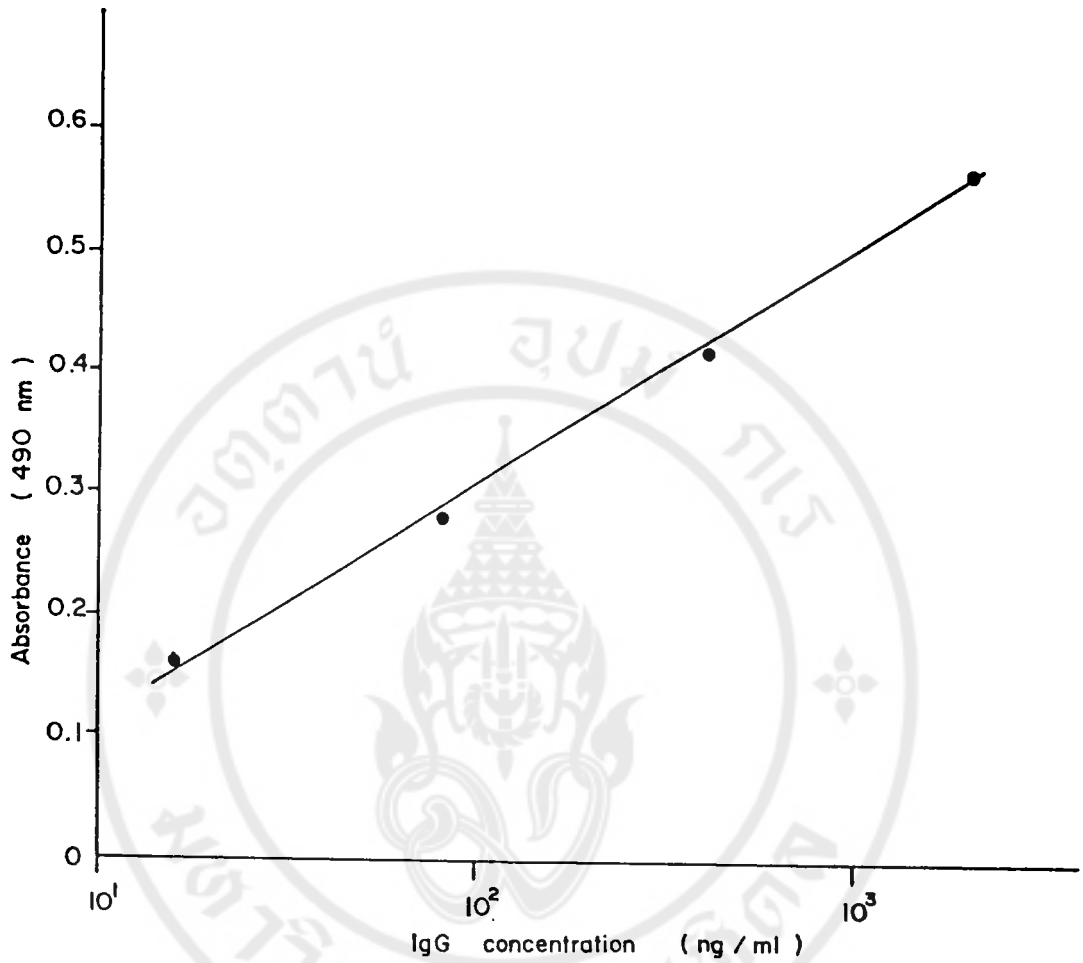


Figure 4 A typical standard curve for quantitation of total IgG levels by ELISA.

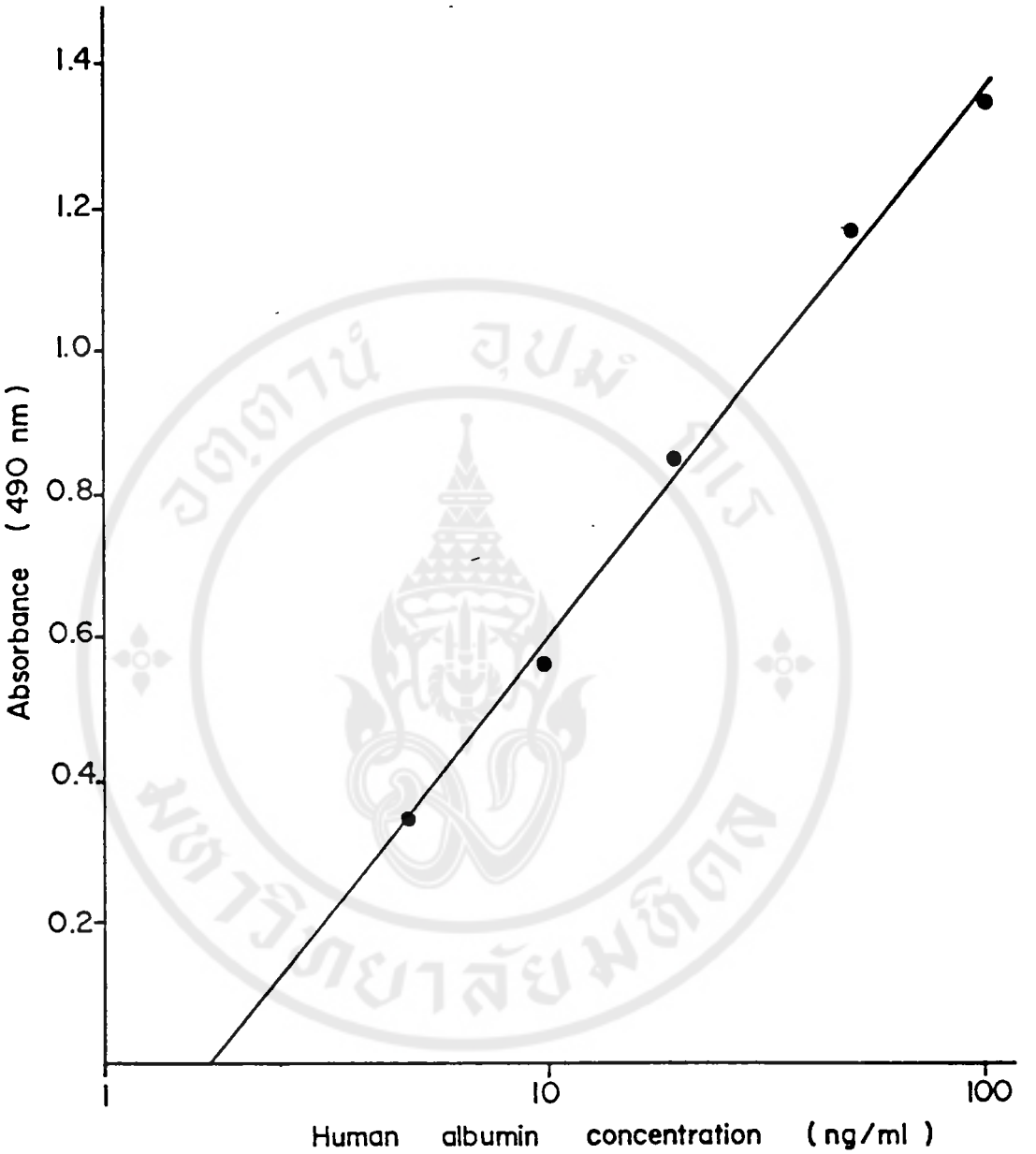


Figure 5 **A typical standard curve for quantitation of albumin levels by ELISA.**

in Section 10.2 A typical standard curve of the quantitation of albumin is shown in Figure 5.

10.4 Protein determination

Protein concentrations were estimated by a standard Folin method (44) except for the surface extract whose protein content was determined by technique modified to minimize some interference from residual detergent in the samples (45). Both techniques used bovine serum albumin (BSA, Sigma) as a standard.

11. Complement-consumption test for detection of immune complexes

The method used for the detection of immune complexes that might be present in the CSF was slightly modified from the method used by Harkiss and Brown (46).

11.1 Precipitation of immune-complexes in CSF by PEG 6000

The immune complexes present in some CSF specimens might be present in quantity too small to be measured directly and must therefore be concentrated prior to being measured. The concentration procedure was done by mixing 50 µl of borate buffer 0.1 M pH 8.4 and 50 µl of 0.2 M EDTA with 300 µl of CSF specimens or heat-aggregated IgG (as a control) and then 100 µl of a 12.5% PEG 6000 was added; the mixture was immediately vortexed and then left standing at 4°C for 90 min. After centrifugation at 5,000 rpm for 10 min at 4°C, the supernatant was discarded and the pellet was washed by the addition of 1.0 ml of 2.5% PEG. The tube was vortexed again to resuspend the pellet and centrifuged at 5,000 rpm for 15 min at 4°C. The supernatant was discarded and the pellet was

finally resuspended in 30 µl of complement fixing diluent (CFD) or veronal buffer (See Appendix). This protocol allowed one to concentrate the immune complexes in the specimens by a factor of 10.

11.2 Complement-consumption and CH_{50} microtitration technique

The concentrated solutions of immune complexes were assayed functionally by measuring their ability to activate complement. Thirty microliters of resuspended immune complexes were added to 30 µl of NHS as a source of complement (NHS was titrated for complement activity before adding). After incubation at 37°C for 30 min, the tubes were placed on ice and residual hemolytic complement was assayed for CH_{50} by microtitration technique. The control tubes containing 30 µl CFD and 30 µl NHS were included in the complement consumption step.

The CH_{50} microtitration technique was modified from the one routinely used at Ramathibodi Hospital (Petchclai, B. personnal communications). In brief, 25 µl of samples to be measured were first diluted with gelatin-veronal buffer (GVB) (see Appendix) and then diluted two-fold to a titer of 1:32 in U-bottom microtiter plates (Cooke Engineering Co., Alexandria, Virginia, U.S.A.). The volume of the mixture in each well was equalized with GVB before 25 µl of a 2.5% sensitized sheep red cells (see Appendix) was added. The plate was shaken before incubation at 37°C for 1 hr in moist chamber. The extent of hemolysis was determined from the optical density value at 570 nm. Mean absorbance value from a duplicate test was used for the calculation of the CH_{50} units.

12. Statistical analysis

To evaluate the statistical significance of data from different groups of patients, the Kruskal-Wallis one-way analysis of variance by ranks followed by multiple comparisons test (47) was used.

CHAPTER IV

RESULTS

1. The kinetics of ES production

The level of ES production by L_3G maintained *in vitro* in BME medium is shown in Figure 1. The quantity of ES produced, as expressed by OD at 280 nm, reached a maximum level during the first few days of incubation, particularly during the first twenty four hours. The levels of ES gradually declined thereafter, and this coincided with the worms becoming smaller, looking paler than the fresh worms, and becoming more and more inactive. It was calculated that each worm secreted approximately 0.7 µg of ES protein per day during the first 4 days of cultivation.

2. Standardization of ELISA for quantitation of L_3G antigens

Optimal concentrations of different rabbit IgG anti-L_3G antigens to be used for coating the microtiter plate were predetermined by checkerboard titration. The titration curves for each IgG anti-*G. spinigerum* antigens were plotted as shown in Figures 6-8. The maximal binding of each antigen to the corresponding specific rabbit IgG antibody was determined and used for the selection of optimal concentrations of IgG antibodies to each antigen in Table 2. These IgG concentrations were used in the remaining portions of this study.

The dilutions of biotinylated IgG antibody to each antigen and streptavidin-conjugated peroxidase were also predetermined by checkerboard titration. The titration curves of both reagents are

shown in Figures 9-14. The optimal dilutions of both reagents for each of the 3 antigen-antibody systems are summerized as shown in Table 3. These dilutions were used in the remaining of this study.

The optimal time required for each reaction was also determined. The most optimal conditions for all interactions was found to be 1 hr at 37°C (Figures 15-17) and this condition was used throughout this study.

The optimization of enzymatic reaction was investigated by allowing the substrate to react with streptavidin peroxidase in the dark at room temperature. The most optimal time was 40 min (Figure 18).

The reproducibility of ELISA for the quantitation of antigen was determined by calculating the mean values and establishing a standard deviations of various L_3G antigens run on different occasions. The mean absorbance of well without antigen was subtracted from the mean absorbance of each antigen concentration and these corrected absorbance values of antigen were plotted against the corresponding concentration as shown in Figures 19-21. The inter-run coefficient of variation of absorbance values was calculated in order to estimate the reproducibility of ELISA test as shown in Tables 4-6.

The sensitivity of the established ELISA was estimated from the dose response curve. It was revealed that the amount of antigen as low as 10 ng/ml, 2 ng/ml and 31.2 ng/ml of somatic extract, ES and surface extract could be detected respectively.

The specificity of ELISA test for the detection of L_3G antigens was evaluated by constructing dose reponse curve using

different parasitic antigens (*O. viverrini, S. mansoni, T. crassiceps, T. hydatigena,* third stage larvae (L_3A) and female adult crude extract of *A. cantonensis*). The results illustrated in Figures 22-24 showed that protein antigens prepared from L_3G had stronger reactivity than those of other parasites. Both somatic and surface systems could detect some of female adult crude extract of *A. cantonensis* if they were tested at very high antigen concentration, but no reactivity of these parasitic antigens in ES system. However, the absorbance value of the heterologous antigens detected by these ELISA are much lower than those of *G. spinigerum* at the same protein concentration.

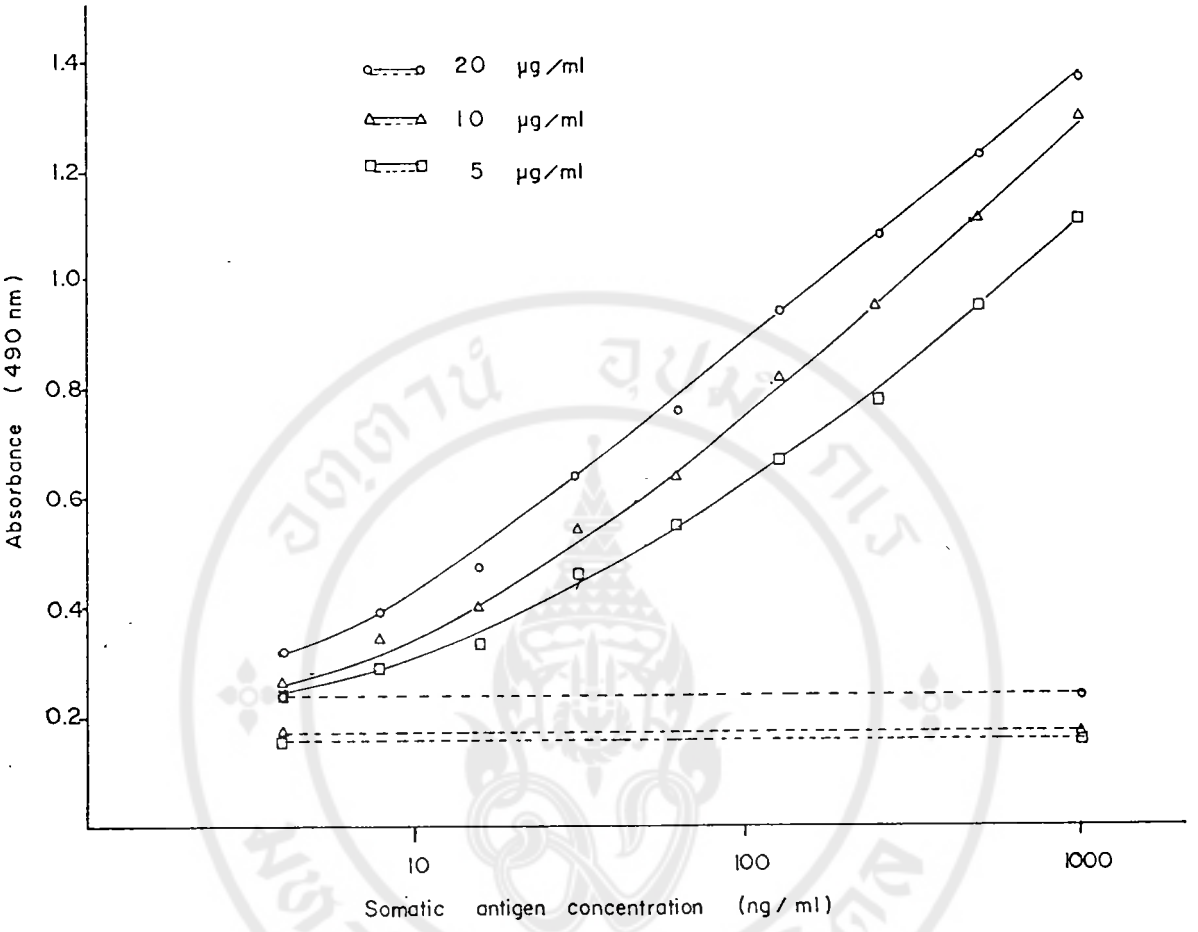


Figure 6 Checkerboard titration of rabbit IgG anti-somatic antigen (20, 10, 5 µg/ml) reacting with various concentrations of somatic antigen (___) and the corresponding titration curves performing simultaneously with uncoated plates (without antigen)(---). Biotinylated IgG anti-somatic antigen and streptavidin-peroxidase were arbitrarily fixed at dilution 1:200 and 1:500,respectively. The incubation time for each step was arbitrarily fixed at 1 hr and the enzymatic reaction was allowed to proceed for 40 min. The working concentration for rabbit IgG anti-somatic to be used in subsequent studies was found to be 10 µg/ml, judging from the highest difference of absorbance reading between the presence and absence of antigen.

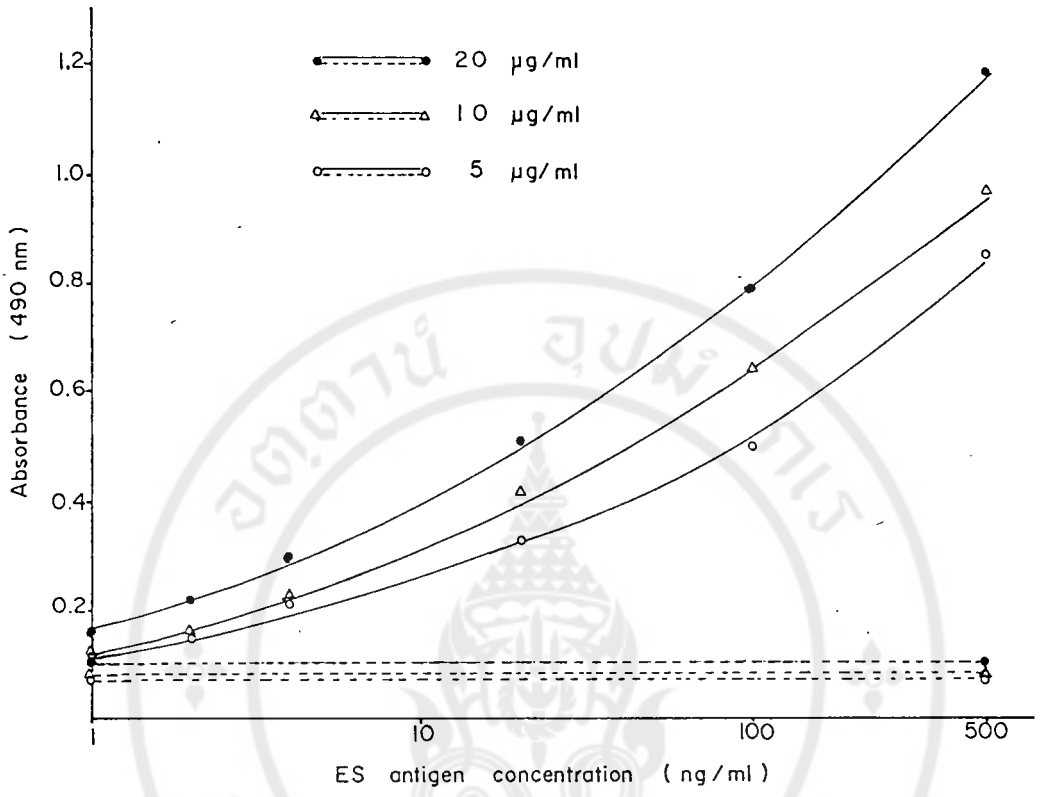


Figure 7 Checkerboard titration of rabbit IgG anti-ES antigen (20, 10, 5 µg/ml) reacting with various concentrations of ES antigen (___) and the corresponding titration curves performing simultaneously with uncoated plates (without antigen)(---). Biotinylated IgG anti-ES antigen and streptavidin-peroxidase were arbitrarily fixed at dilution 1:100 and 1:2,000, respectively. The incubation time for each step was arbitrarily fixed at 1 hr and the enzymatic reaction was allowed to proceed for 40 min. The working concentration for rabbit IgG anti-ES to be used for coating plate was found to be 20 µg/ml, judging from the highest difference of absorbance reading between the presence and absence of antigen.

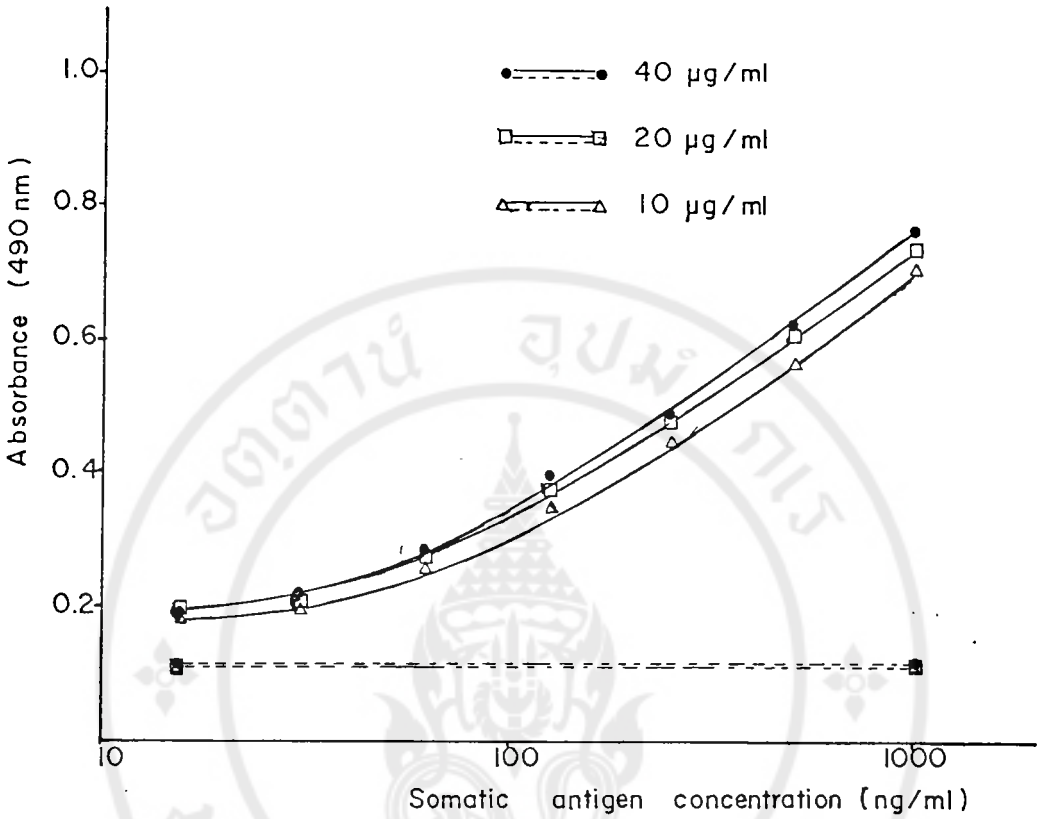


Figure 8 Checkerboard titration of rabbit IgG anti-surface antigen (10, 20, 40 µg/ml) reacting with various concentrations of somatic antigen (___) and the corresponding titration curves performing simultaneously with uncoated plates (without antigen)(---). Biotinylated IgG anti-somatic antigen and streptavidin-peroxidase were arbitrarily fixed at dilution 1:200 and 1:1,000, respectively. The incubation time for each step was arbitrarily fixed at 1 hr and the enzymatic reaction was allowed to proceed for 40 min. The working concentration for rabbit IgG anti-surface to be used in subsequent studies was found to be 20 µg/ml, judging from the highest difference of absorbance reading between the presence and absence of antigen.

Table 2 Optimal concentrations of rabbit IgG anti-L_3G to be used for the quantitation of specific antigens by ELISA.

Antigen to be tested	Optimal concentration (µg/ml)
somatic extract	10.0
ES	20.0
surface extract	20.0

Figure 9 Optimization of biotinylated rabbit IgG anti-somatic antigen. The plate was coated with 50 µl of rabbit IgG anti-somatic antigen at concentration of 10 µg/ml and allowed to react with various concentrations of somatic antigen (___) or diluent alone (without antigen) (---). After incubation, the absorbed antigen was detected by biotin-streptavidin system. The stock biotinylated rabbit IgG anti-somatic antigen were used at arbitrary dilution of 1:100, 1:200 and 1:400. Streptavidin-conjugated peroxidase was arbitrarily fixed at 1:500. The incubation times were fixed as previously described. The most suitable working dilution of biotinylated rabbit IgG anti-somatic to be used for detecting the antigen in CSF appears to be around 1:200, judging from the highest difference of absorbance reading between the presence and absence of antigen.

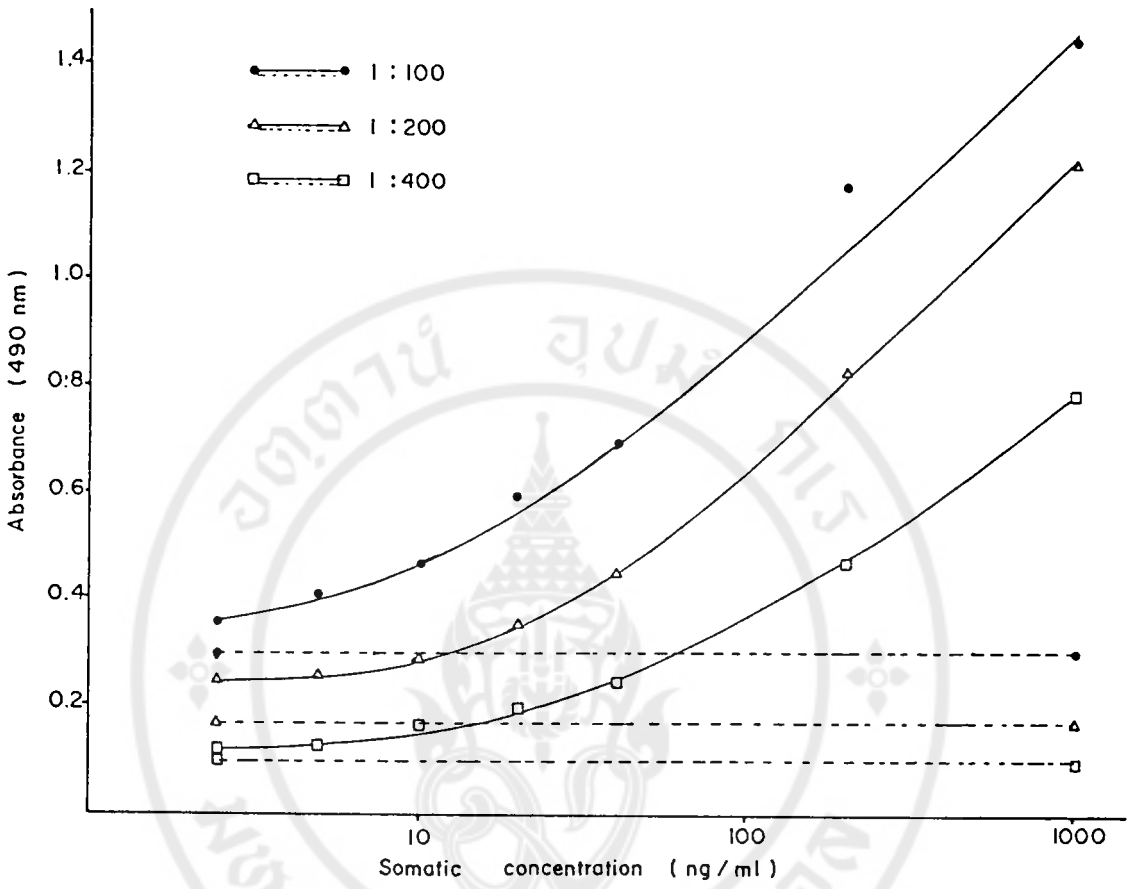


Figure 10 Optimization of biotinylated rabbit IgG anti-ES antigen. The plate was coated with 50 µl of rabbit IgG anti-ES antigen at a concentration of 20 µg/ml and allowed to react with various concentrations of ES antigen (___) or diluent alone (without antigen) (---). After incubation, the absorbed antigen was detected by biotin-streptavidin system. The stock biotinylated rabbit IgG anti-ES antigen were used at arbitrary dilutions of 1:100, 1:200 and 1:400. Streptavidin-conjugated peroxidase was fixed at 1:2,000. The incubation time was fixed as previously described. The most suitable working dilution of biotinylated rabbit IgG anti-ES to be used for detecting with antigen in CSF appears to be around 1:200, judging from the highest difference of absorbance reading between the presence and absence of antigen.

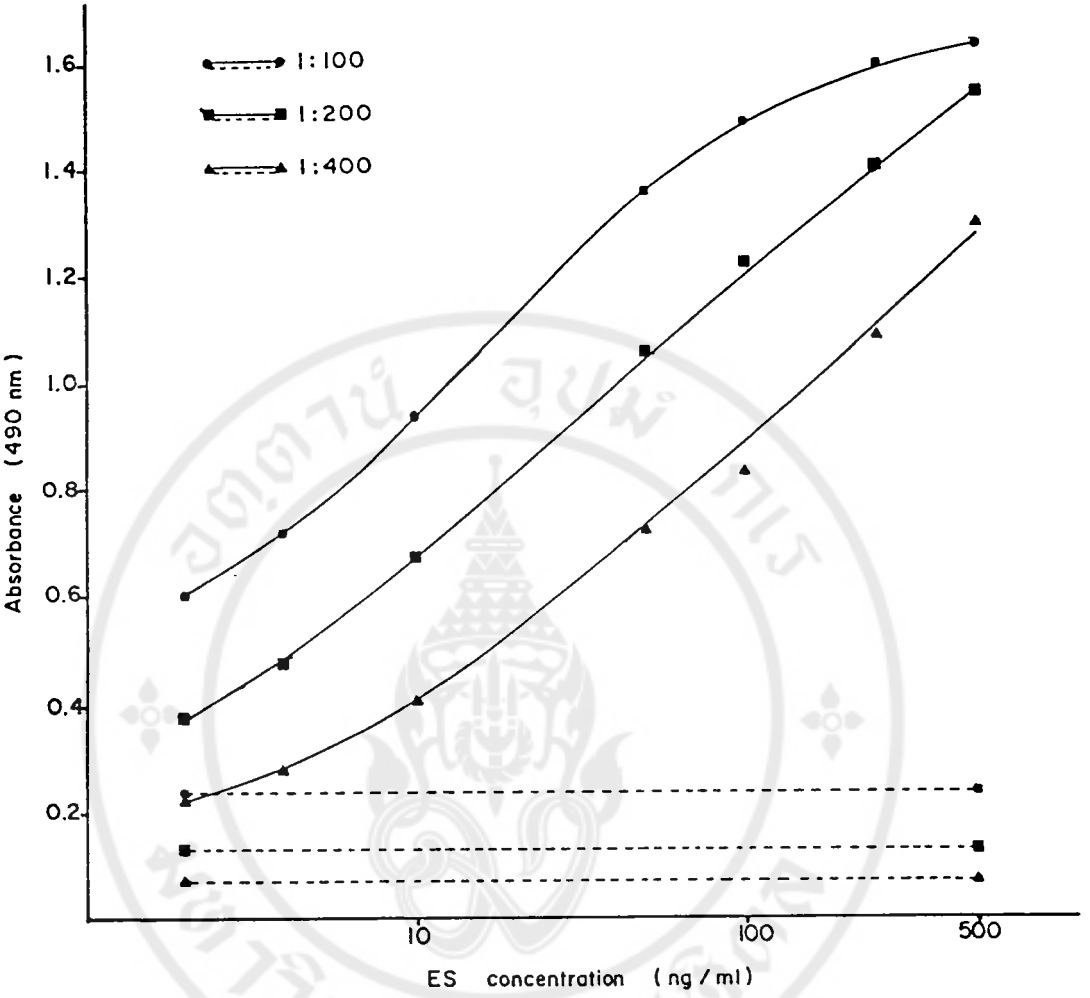


Figure 11 Optimization of biotinylated rabbit IgG anti-surface antigen. The plate was coated with 50 µl of rabbit IgG anti-surface antigen at a concentration of 20 µg/ml and allowed to react with various concentrations of somatic antigen (___) or diluent alone (without antigen) (---). After incubation, the absorbed antigen was detected by biotin-streptavidin system. The stock biotinylated rabbit IgG anti-ES antigen were used at arbitrary dilution of 1:100, 1:200 and 1:400. Streptavidin-conjugated peroxidase was arbitrarily fixed at 1:1,000. The incubation times were fixed as previously described. The most suitable working dilution of biotinylated rabbit IgG anti-surface to be used for detecting the antigen in CSF appears to be around 1:200, judging from the highest difference of absorbance reading between the presence and absence of antigen.

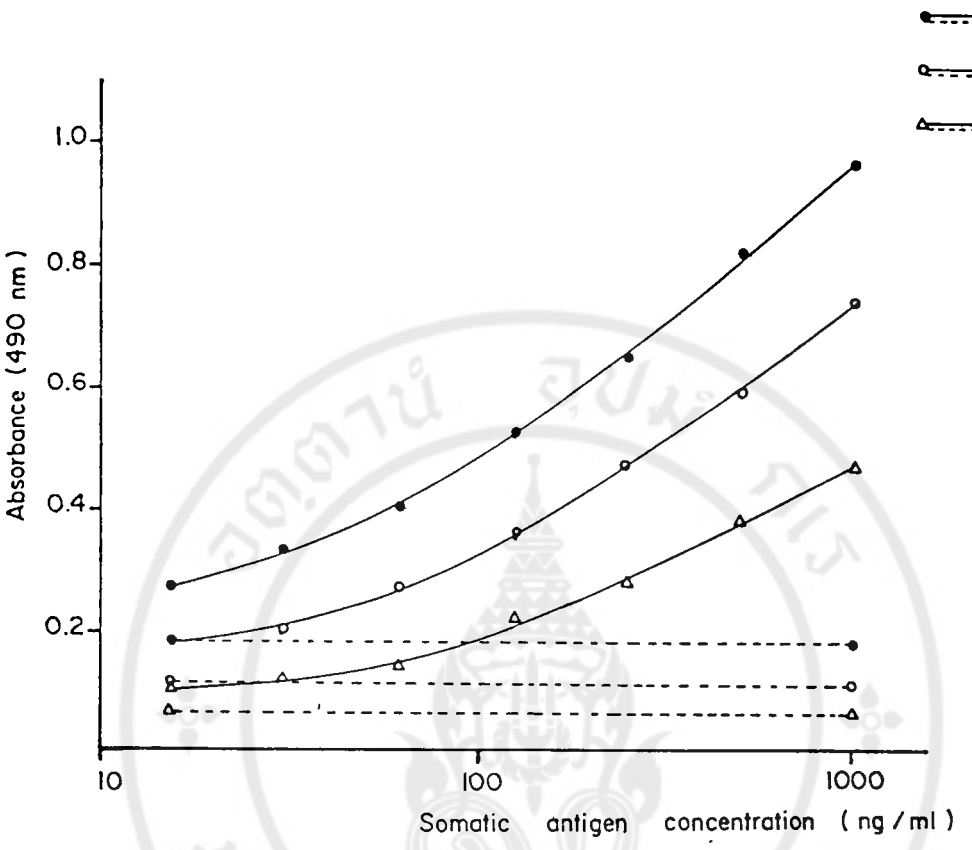


Figure 12 Optimization of streptavidin-conjugated peroxidase reacting with the biotinylated IgG anti-somatic antigen. The plate was coated with 50 µl of rabbit IgG anti-somatic antigen at a concentration of 10 µg/ml and allowed to react with various concentrations of somatic antigen (___) or diluent alone (without antigen) (---). A 50 µl of a 1:200 biotinylated IgG anti-somatic was used to detect the absorbed antigen. A stock solution of streptavidin-conjugated peroxidase was used at arbitrary dilution of 1:500, 1:1,000 and 1:2,000. The incubation time was fixed as previously described. The most suitable working dilution of streptavidin-conjugated peroxidase to be used to detect the biotinylated IgG anti-somatic appears to be around 1:1,000, judging from the highest difference of absorbance reading between the presence and absence of antigen.

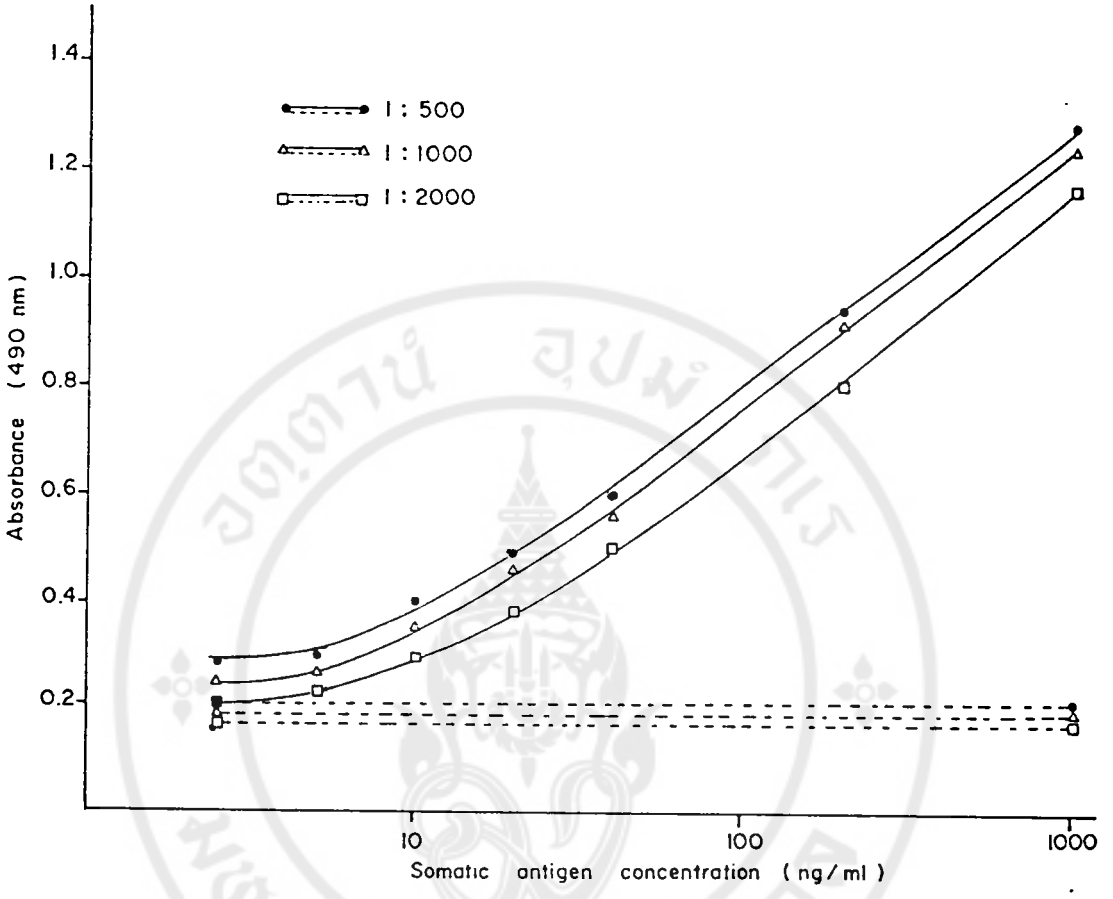


Figure 13 Optimization of streptavidin-conjugated peroxidase reacting with the biotinylated IgG anti-ES antigen. The plate was coated with 50 µl of rabbit IgG anti-somatic antigen at a concentration of 20 µg/ml and allowed to react with various concentrations of ES antigen (___) or diluent alone (without antigen)(---). A 50 µl of a 1:200 biotinylated IgG anti-ES was used to detect the absorbed antigen. A stock solution of streptavidin-conjugated peroxidase were used at arbitrary dilution of 1:1,000, 1:2,000 and 1:4,000. The incubation time was fixed as previously described. The most suitable working dilution of streptavidin-conjugated peroxidase to be used to detect the biotinylated IgG anti-ES appears to be around 1:2,000, judging from the highest difference of absorbance reading between the presence and absence of antigen.

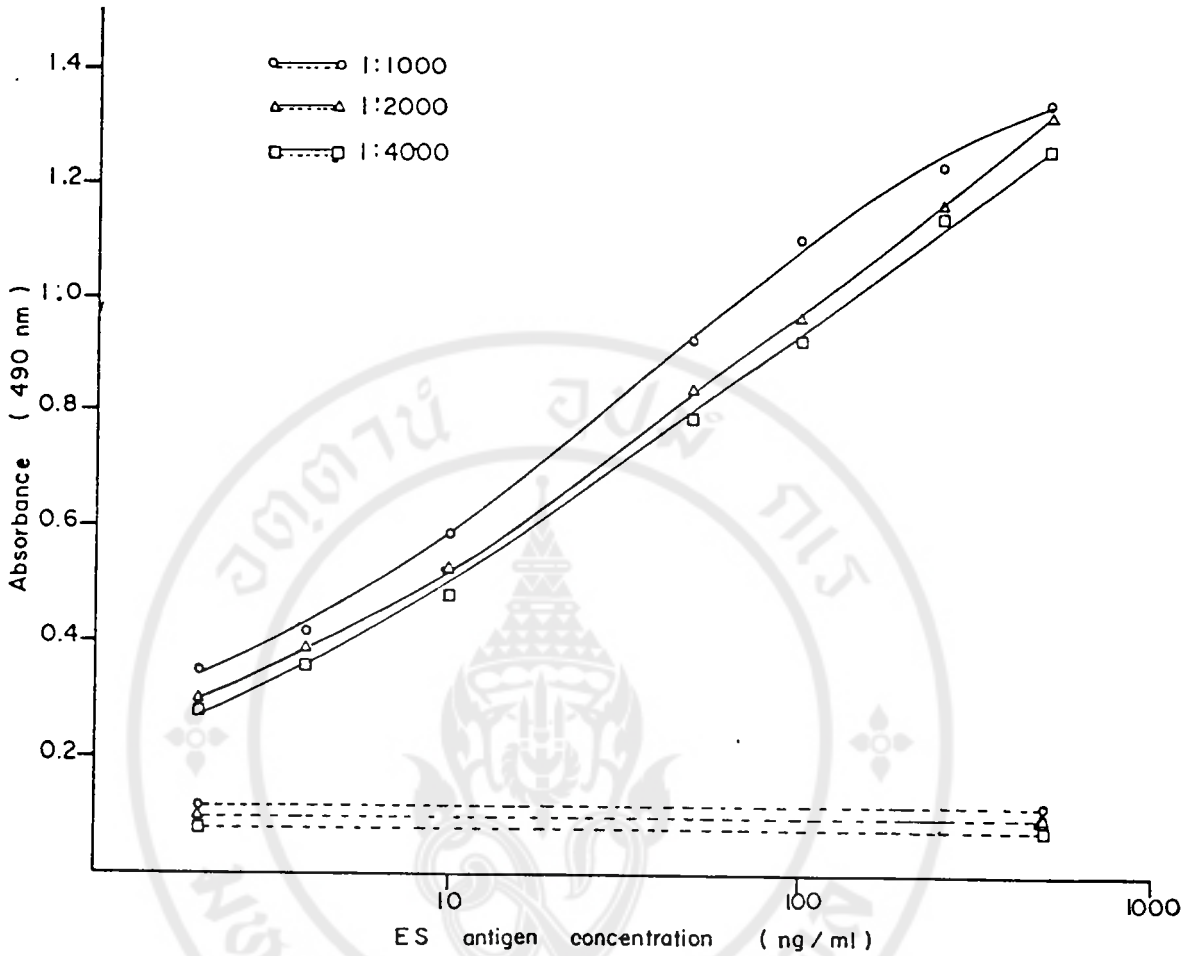


Figure 14 Optimization of streptavidin-conjugated peroxidase reacting with the biotinylated IgG anti-surface antigen. The plate was coated with 50 µl of rabbit IgG anti-surface antigen at a concentrations 20 µg/ml and allowed to react with various concentration of somatic antigen (___) or diluent alone (without antigen)(---). A 50 µl of a 1:200 biotinylated IgG anti-surface was used to detect the absorbed antigen. A stock solution of streptavidin-conjugated peroxidase were used at arbitrary dilution of 1:500, 1:1,000 and 1:2,000. The incubation time was fixed as previously described. The most suitable dilution of streptavidin-conjugaed peroxidase to be used to detect the biotinylated IgG anti-surface appears to be around 1:1,000, judging from the highest difference of absorbance reading between the presence and absence of antigen.

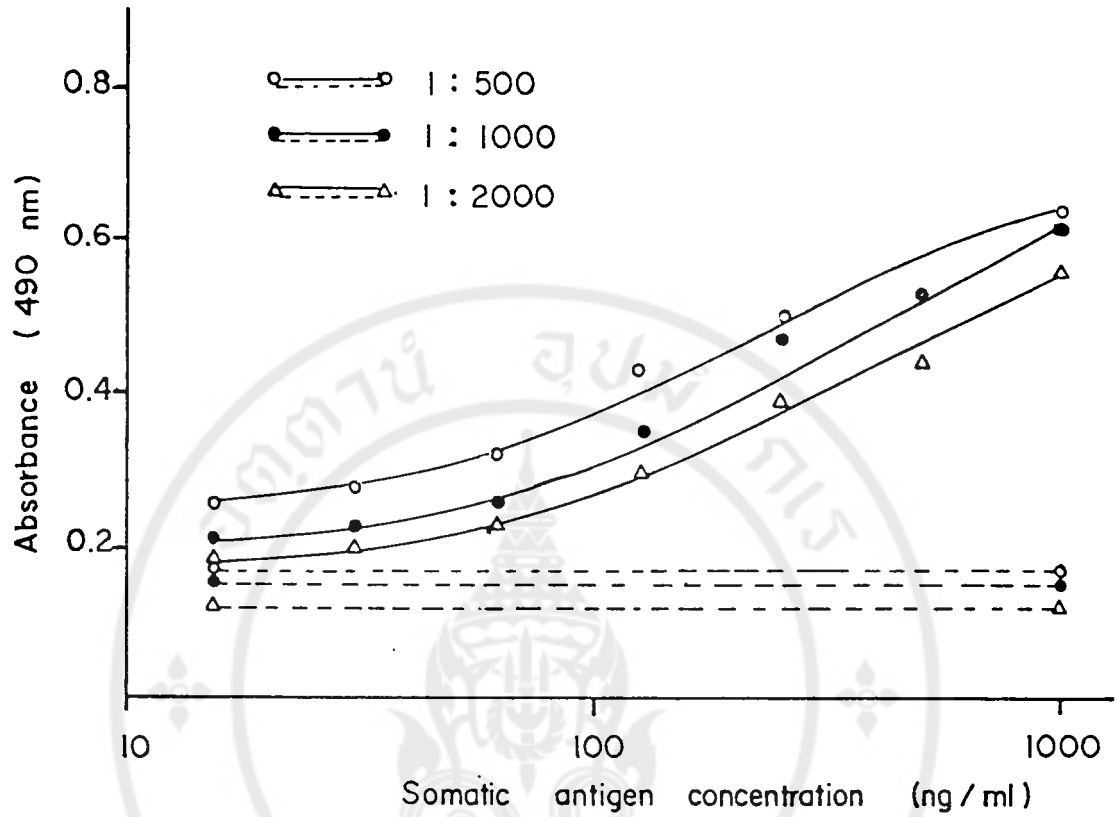


Table 3 Optimal dilutions of biotinylated IgG anti-L_3G and streptavidin-conjugated peroxidase to be used for the determination of specific antigens by ELISA.

Antigen in the system	Optimal dilution of corresponding	
	biotinylated IgG	streptavidin-conjugated peroxidase
somatic	1:200	1:1,000
ES	1:200	1:2,000
surface	1:200	1:1,000

Figure 15 Determination of optimal time for the interaction between rabbit IgG anti-somatic and somatic antigen. The plate was coated with 50 µl of a 10 µg/ml rabbit IgG anti-somatic and allowed to react with various concentrations of somatic antigen (___) and the corresponding titration curves performing simultaneously with diluent (without antigen)(---). The incubation time in this step varied from 1 to 3 hr at 37°C. The working dilution of other reagents was predetermined as described above. The incubation times for other steps were fixed at 1 hr and the enzymatic reaction was allowed to take place for 40 min at room temperature in the dark. The optimal time for this interaction was found to be 1 hr, judging from the shortest incubation time which gave maximal reaction performing under optimal concentrations of reagents.

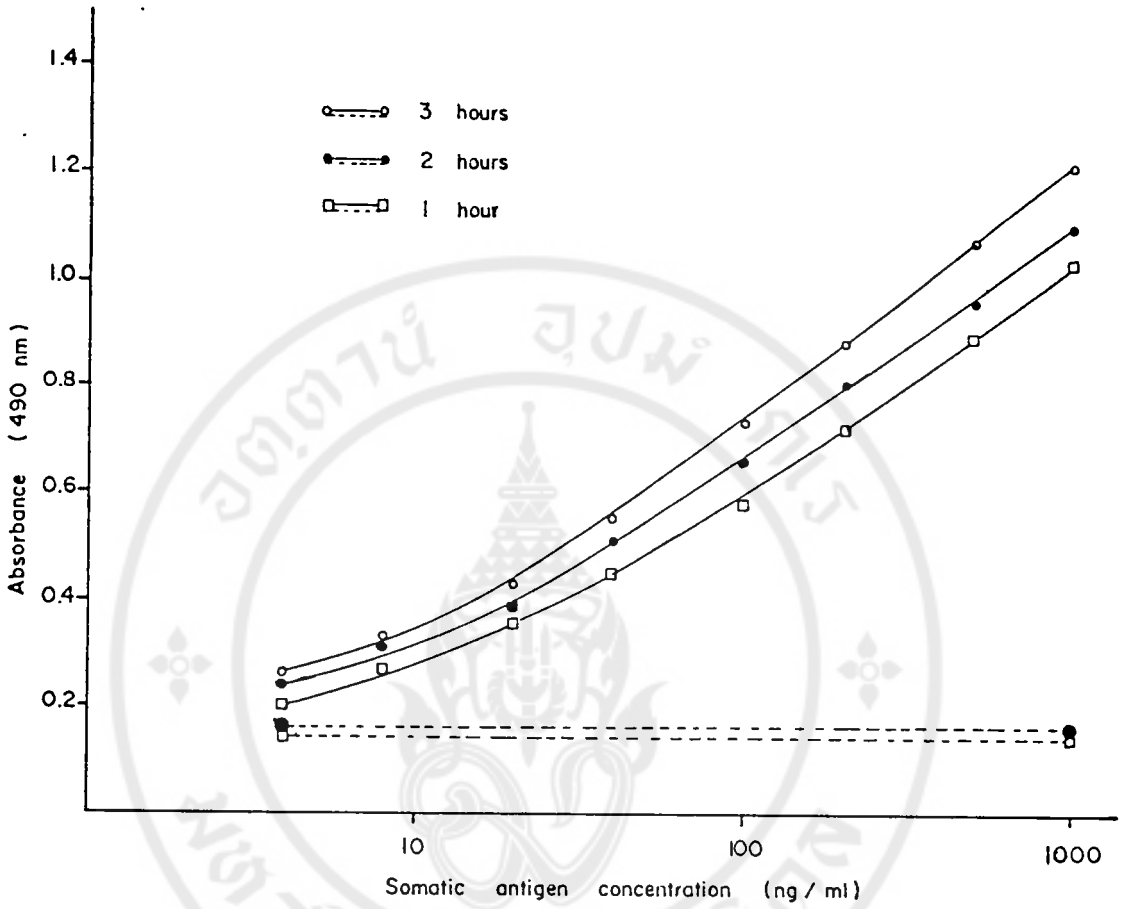


Figure 16 Determination of optimal time for the interaction between biotinylated IgG anti-somatic and absorbed somatic antigen. The plate was coated with 50 µl of a 10 µg/ml rabbit IgG anti-somatic and allowed to react with various concentrations of somatic antigen (___) and the corresponding titration curves performing simultaneously with diluent (without antigen) (---) at 37°C for 1 hr. A 50 µl of 1:200 biotinylated IgG anti-somatic antigen was allowed to react with absorbed antigen. The incubation time in this step varied from 30 to 120 min at 37°C. A 50 µl of 1:1,000 streptavidin-conjugated peroxidase was used to react with the biotinylated IgG anti-somatic at 37°C for 1 hr and the enzymatic reaction was allowed to take place for 40 min at room temperature in the dark. The optimal time for this interaction was found to be 60 min, judging from the shortest incubation time which gave maximal reaction performing under optimal concentrations of reagents.

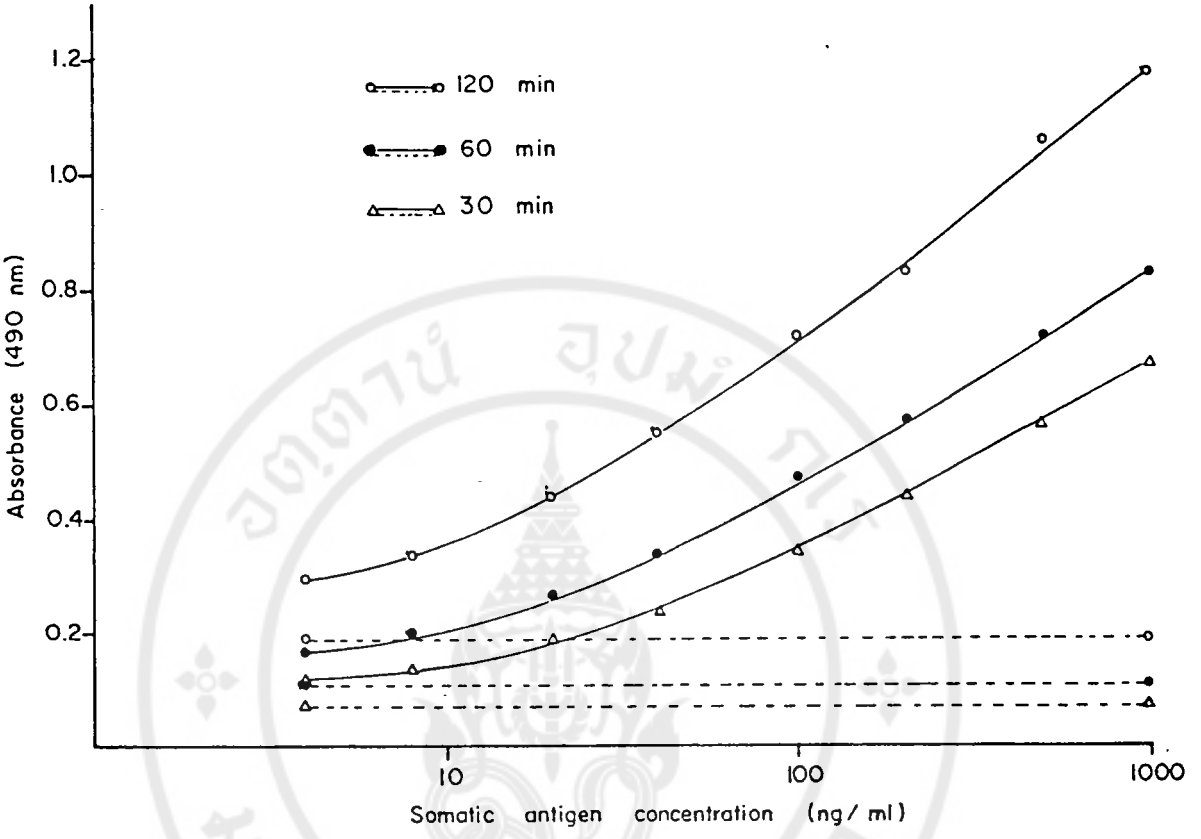
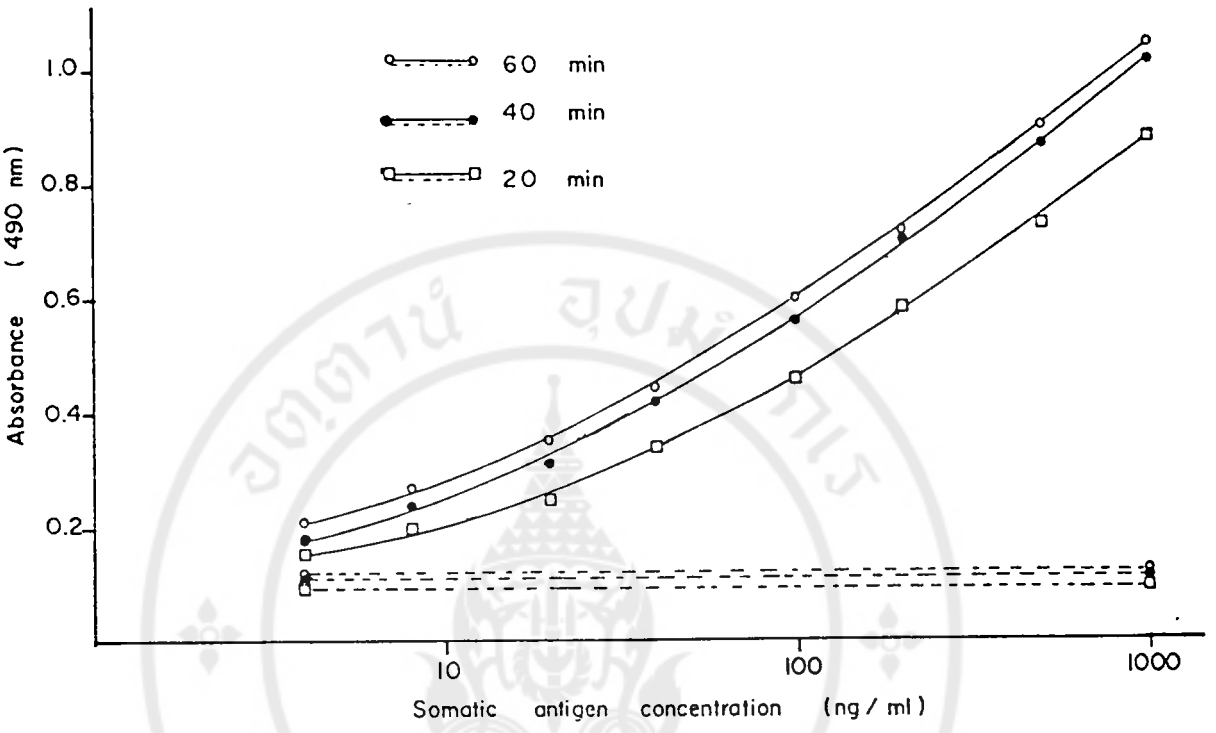


Figure 17 Determination of optimal time for the interaction between biotinylated IgG anti-somatic and streptavidin-conjugated peroxidase. The plate was coated with 50 µl of a 10 µg/ml rabbit IgG anti-somatic and allowed to react with various concentrations of somatic antigen (___) and the corresponding titration curves performing simultaneously with diluent (without antigen) (---) for 1 hr at 37°C. A 50 µl of 1:200 biotinylated IgG anti-somatic antigen was allowed to react with the absorbed antigen for 1 hr at 37°C. A 50 µl of 1:1,000 streptavidin-conjugated peroxidase was used to react with the biotinylated IgG anti-somatic. The incubation time in this step varied from 20 to 60 min at 37°C and the enzymatic reaction was allowed to take place for 40 min at room temperature in the dark. The optimal time for this interaction was found to be 60 min, judging from the shortest incubation time which gave maximal reaction performing under optimal concentrations of reagents.



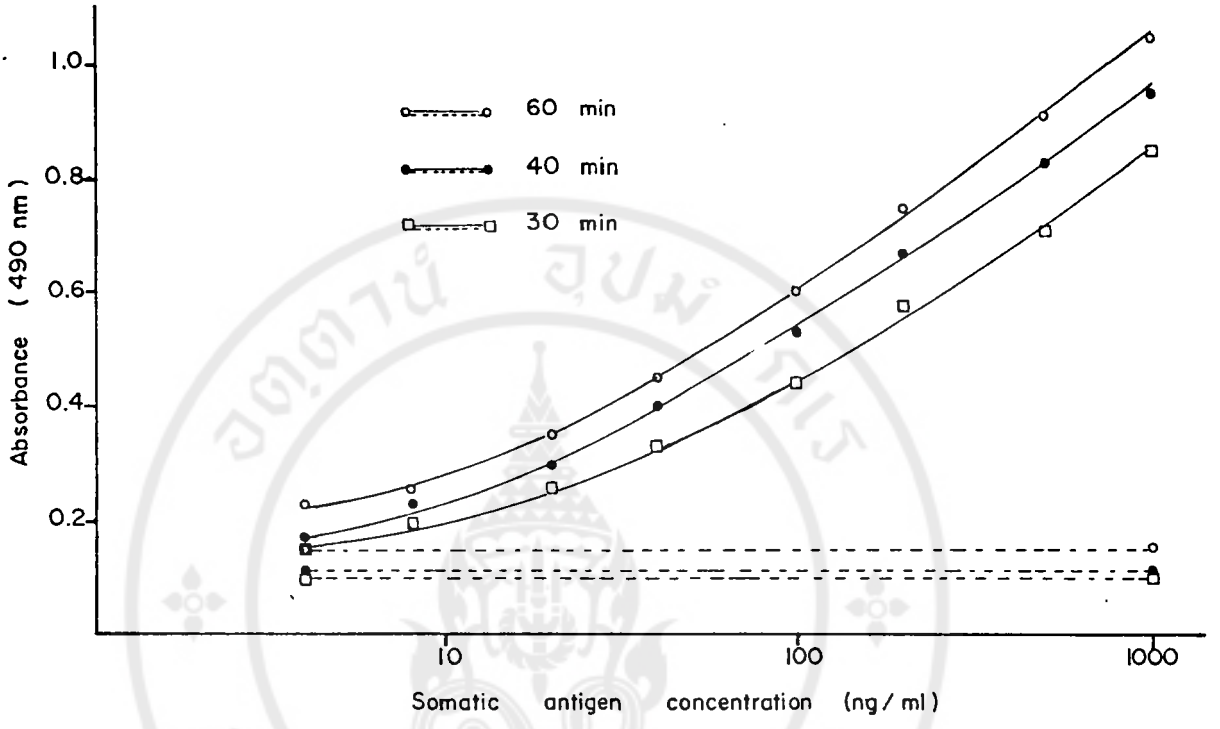


Figure 18 Optimization of enzymatic reaction for the quantitation of somatic antigen. Plate was coated with 50 µl of rabbit IgG anti-somatic antigen at a concentration of 10 µg/ml reacting with various concentrations of somatic antigen (___) and diluent (without antigen) (---) at 37°C for 1 hr. The predetermined working dilutions and incubation times of other steps were performed as previously described. The enzymatic reaction varied from 30 to 60 min at room temperature in the dark. The optimal time for the enzymatic reaction was found to be 40 min.

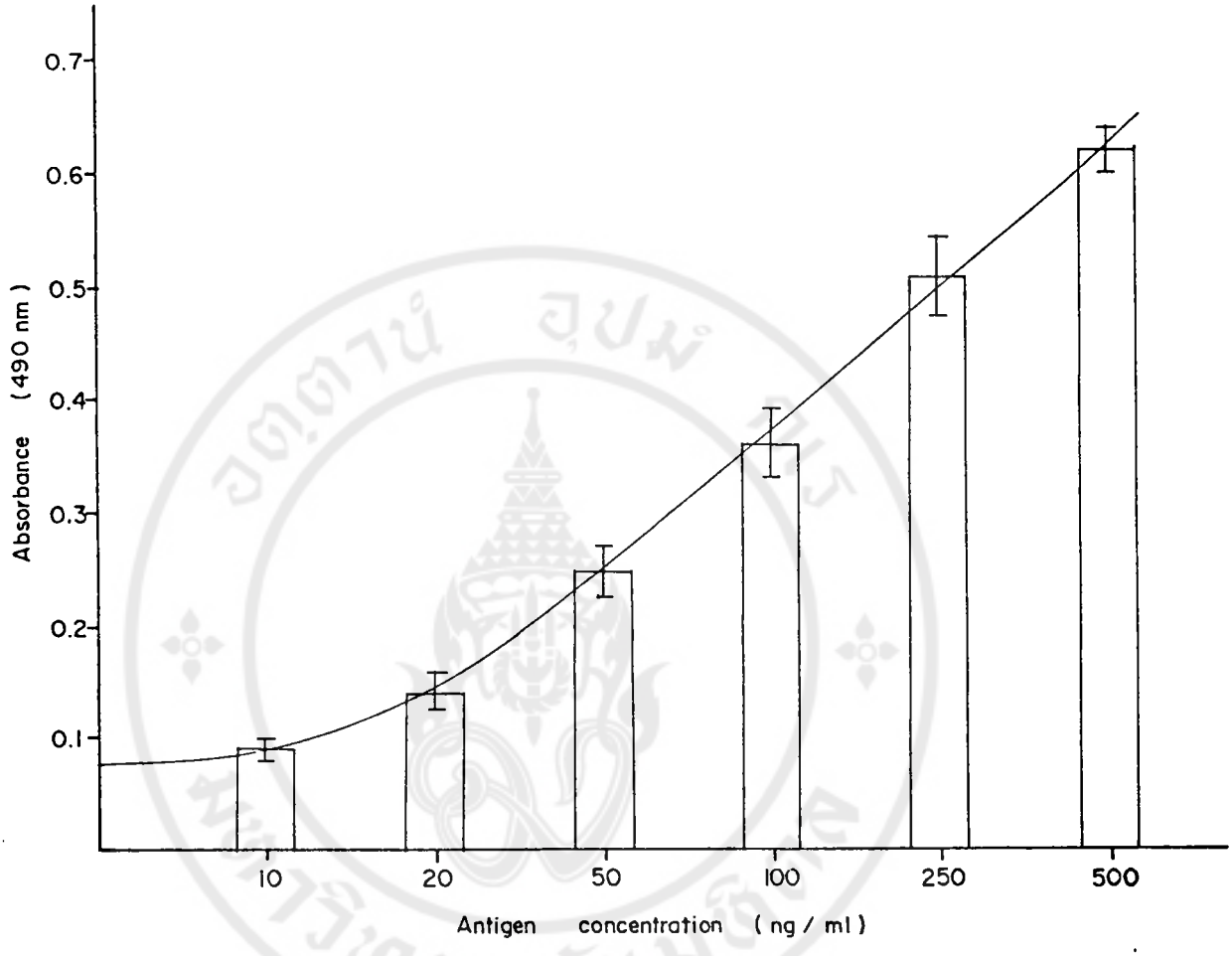


Figure 19 A typical dose response curve for detection of somatic antigen. The distribution of absorbance values together with means and SD of each somatic antigen concentration ranging from 10.0 to 500.0 ng/ml.

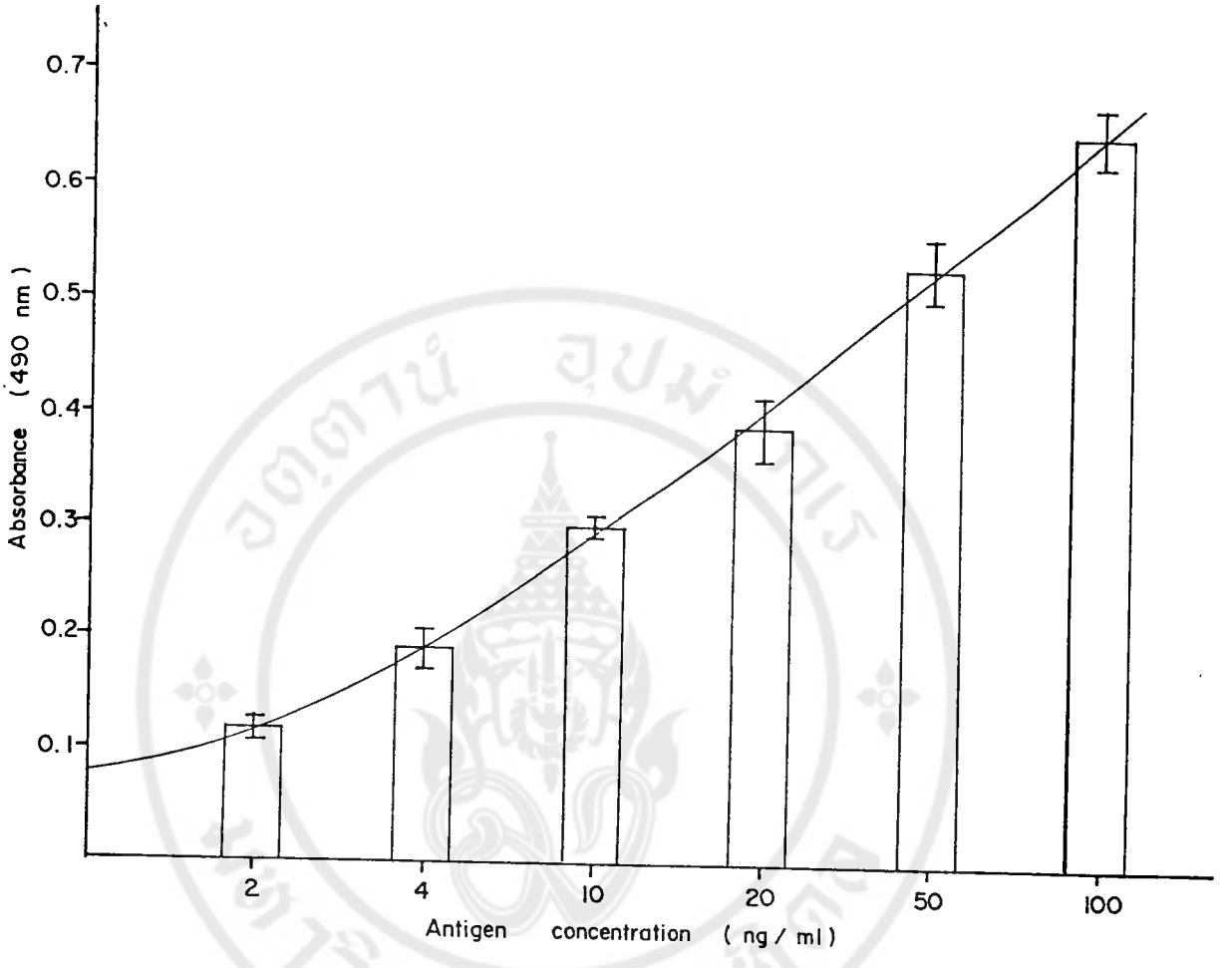


Figure 20 A typical dose response curve for detection of ES antigen. The distribution of absorbance values together with means and SD of each ES antigen concentration ranging from 2.0 to 100.0 ng/ml.

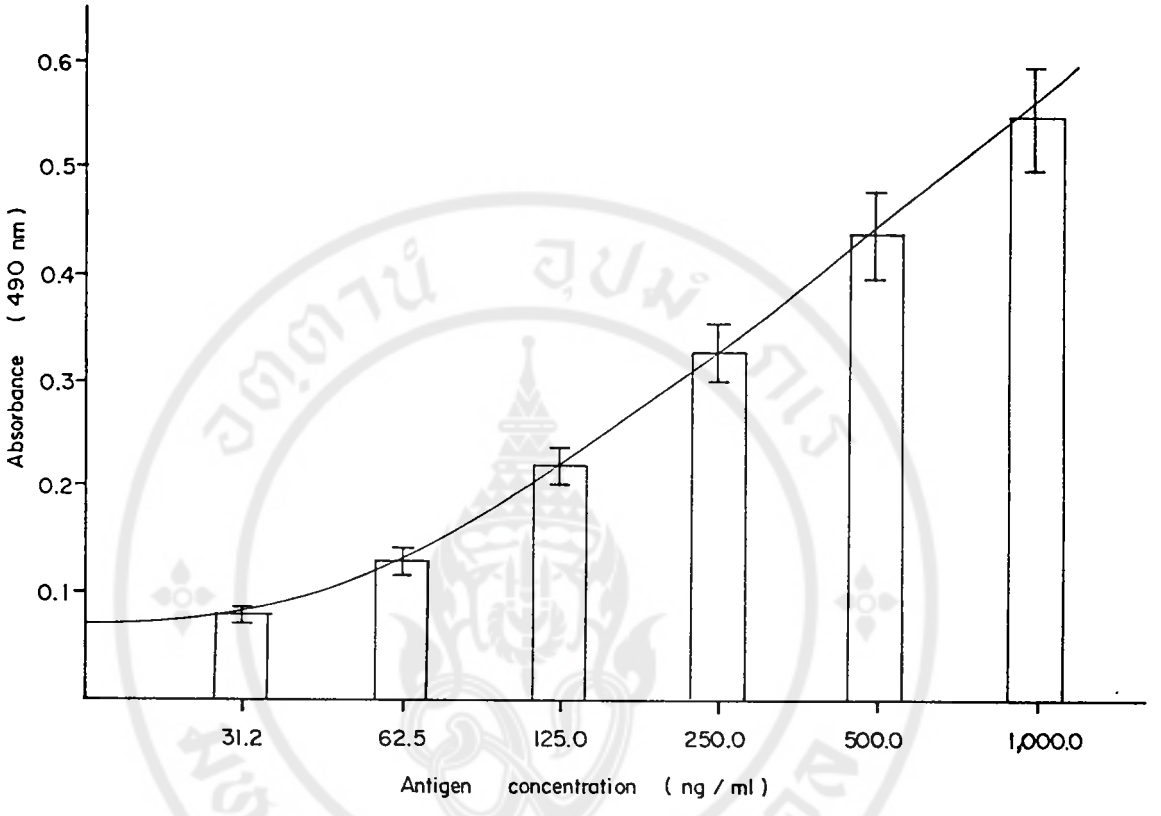


Figure 21 A typical dose response curve for detection of surface antigen. The distribution of absorbance values together with means and SD of each surface antigen concentration ranging from 31.2 to 1,000.0 ng/ml.

Table 4 Inter-run reproducibility of method for quantitation of somatic antigen.

Antigen concentration (ng/ml)	Absorbance values $\bar{x}$ (SD)	% CV
10.0	0.09 (0.009)	10.0
20.0	0.14 (0.013)	9.3
50.0	0.25 (0.022)	8.8
100.0	0.36 (0.028)	7.8
250.0	0.51 (0.033)	6.4
500.0	0.62 (0.02)	3.3

$\bar{x}$ = Arithmetic mean of corrected absorbance values (OD of sample-OD of reagent blank).

SD = Standard deviation of corrected absorbanced values.

CV = Coefficient of variation of corrected absorbance values.

Table 5 Inter-run reproducibility of method for quantitation of ES antigen.

Antigen concentration (ng/ml)	Absorbance values $\bar{x}$	(SD)	% CV
1.0	0.075	(0.017)	22.6
2.0	0.12	(0.008)	6.6
4.0	0.19	(0.017)	8.9
10.0	0.3	(0.011)	3.7
20.0	0.39	(0.027)	7.0
50.0	0.53	(0.027)	5.1
100.0	0.65	(0.023)	3.5

Table 6 Inter-run reproducibility of method for quantitation for surface antigen.

Antigen concentration* (ng/ml)	Absorbance values $\bar{x}$ (SD)	% CV
31.2	0.08 (0.007)	8.8
62.5	0.13 (0.013)	10.0
125.0	0.22 (0.018)	8.2
250.0	0.33 (0.028)	8.5
500.0	0.44 (0.039)	8.8
1,000.0	0.55 (0.05)	9.1

*Used somatic extract antigen as a standard of surface extract.

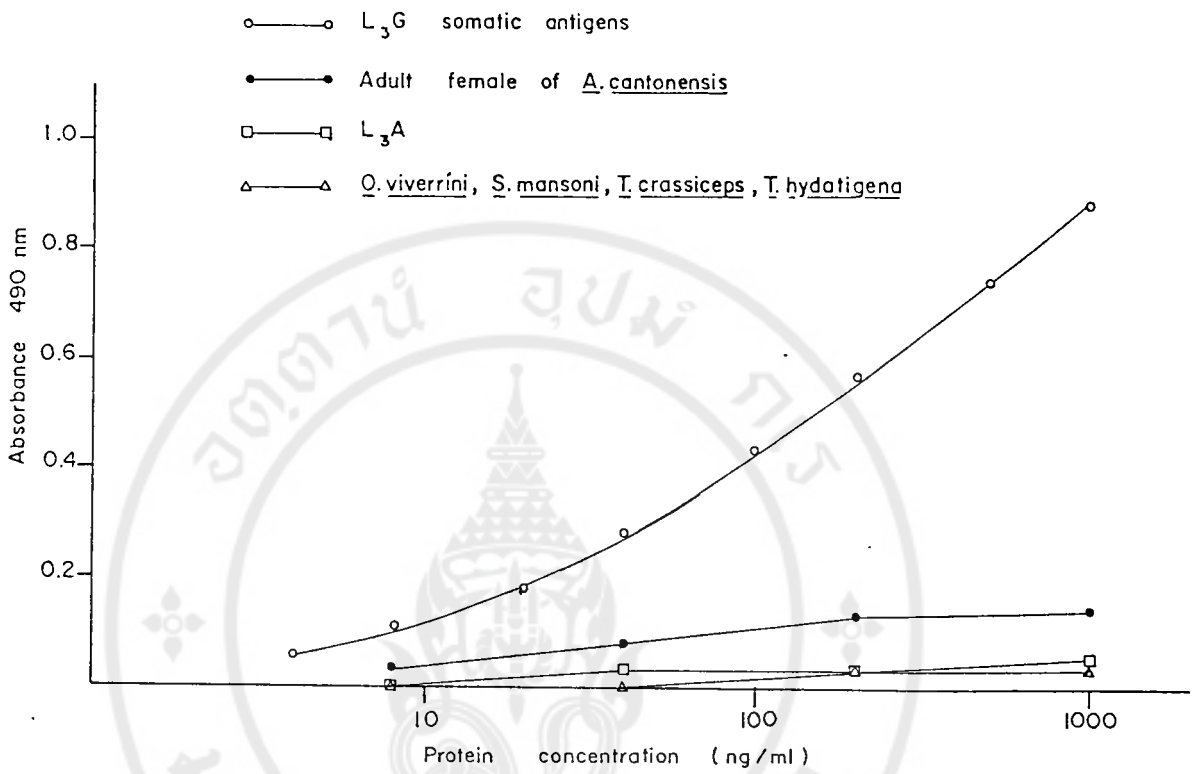


Figure 22 Dose response curves constructed with rabbit IgG anti-L_3G somatic antigen reacted with protein antigens prepared from different parasites.

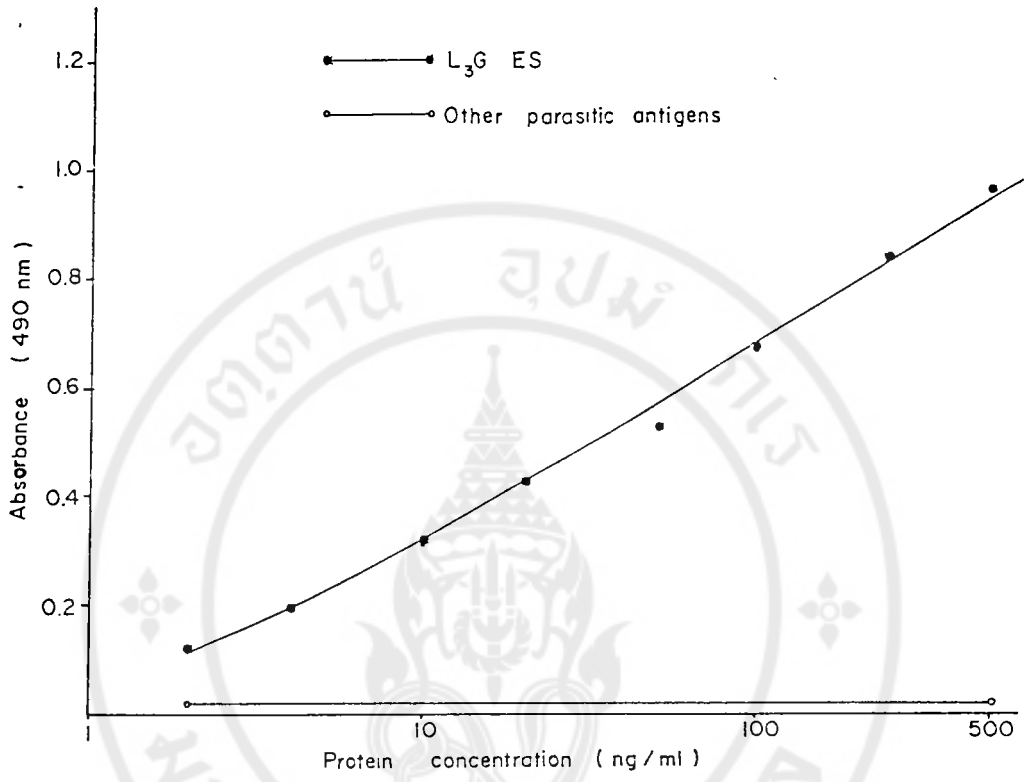


Figure 23 Dose response curves constructed with rabbit IgG anti-L_3G ES antigen reacted with protein antigens prepared from different parasites.

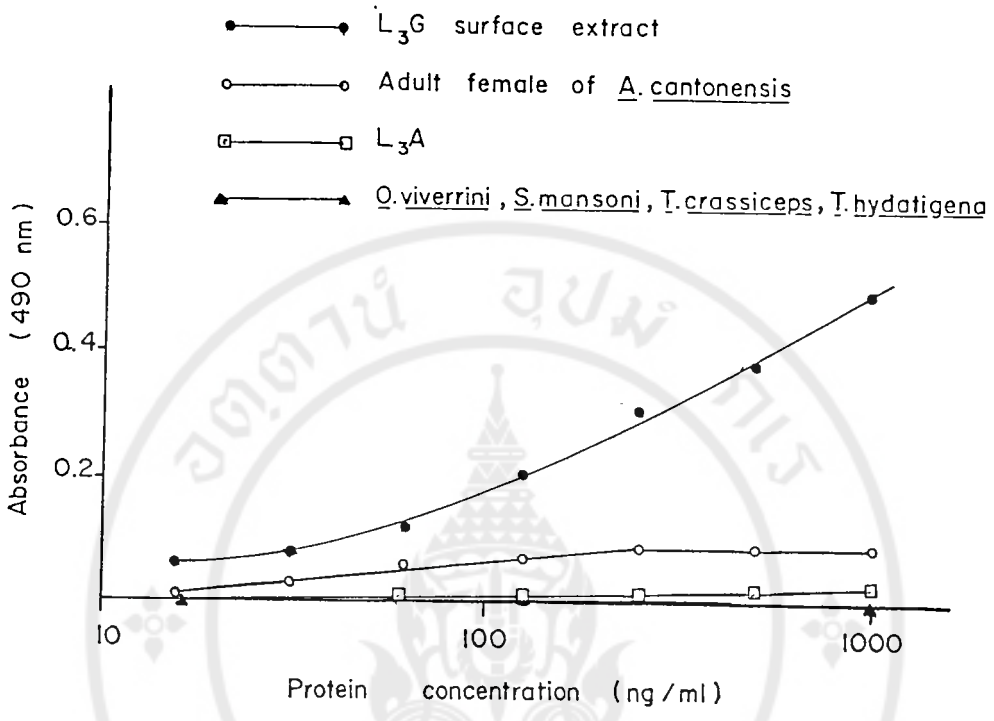


Figure 24 Dose response curves constructed with rabbit IgG anti-L_3G surface antigen reacted with different parasites.

3. Assay of specimens

Because both serum and CSF specimens were available in limited quantity, only some of these specimens were selected to use for assay in this study.

3.1 Quantitation of L_3G antigens in CSF

Six groups of CSF samples were used for the quantitation of somatic and ES antigens, i.e.,

Group I: patient numbers 1-5 with clinical manifestations suggestive of cerebral gnathostomiasis (CNS-G)

Group II: patient numbers 6 and 7 with subarachnoidal haemorrhage (SAH)

Group III: patient numbers 8-11 with eosinophilic meningitis (EM)

Group IV: patient numbers 12-14 with unspecified meningitis (UM)

Group V: patient numbers 15-22 with other CNS involvements

Group VI: patient numbers 29-32 with unspecified illnesses. However, in this group of patients, the physicians sending these specimens must have already some clues of gnathostomiasis and sent specimens just to confirm his or her presumptive diagnosis.

The undiluted CSF samples were assayed for the presence of ES antigen by the conventional B-SA ELISA as described in Materials and Methods. The results obtained from this assay are summerized in Tables 7-12. Using the criteria described in Materials and Methods the B-SA ELISA could detect ES antigen in patient numbers 4, 6, 9, 14, 30 and 32. However, when the ELISA blocking test was performed to confirm the presence of ES antigen by using the 50% inhibition as a criterion, only the patient number 6 in Table 8 showed positive

inhibition test. As a result, the other samples with positive by B-SA ELISA were considered to be negative for *Gnathostoma* ES antigen. This false positive reaction by the conventional B-SA ELISA may have been caused by the presence of non-specific material in CSF samples which can bind with the rabbit IgG anti-ES used for coating the plates.

Due to a limited quantity of some CSF samples, the assay for somatic antigen could be performed only in a few specimens (Tables 7-12). Although the conventional B-SA ELISA could detect the presence of somatic antigen in patient numbers 7, 8,and 30, ELISA blocking test failed to confirm the presence of these antigens and, therefore, the positive B-SA ELISA was considered to be a false positive.

The result from previous study of ES production showed that each worm secreted ES approximately 700 ng/day. Because the total volume of human CSF is about 140 to 150 ml (48), the average concentration of ES produced and secreted into the CSF would be approximately 5 ng/ml per worm per day. Although the sensitivity of the B-SA ELISA used for the detection ES antigen in this study is as low as 2 ng/ml, only one CSF specimen was found to be positive for *Gnathostoma* ES antigen.

3.2 Quantitation of albumin, total IgG and specific IgG to L_3G somatic antigen in serum and CSF

In order to obtain evidence regarding the possibility of having local antibody synthesis within the CNS, specimens from patients with paired serum and CSF in sufficient quantity were analysed for their IgG and albumin content as well as the levels of

Table 7 Assay for *Gnathostoma* ES and somatic antigens in CSF of patients with clinical manifestations suggestive of cerebral gnathostomiasis (CNS-G).

Patient No.	B-SA for ES antigen	ES blocking test	B-SA for somatic antigen	somatic blocking test
1	-	ND*	-	ND
2	-	ND	-	ND
3	-	ND	-	ND
4	+	-	-	ND
5	-	ND	-	ND

* not determined

Table 8 Assay for *Gnathostoma* ES and somatic antigens in CSF of patients with subarachnoidal haemorrhage (SAH).

Patient No.	B-SA for ES antigen	ES blocking test	B-SA for somatic antigen	somatic blocking test
6	+	+	-	ND
7	-	ND*	+	-

* not determined

Table 9 Assay for *Gnathostoma* ES and somatic antigens in CSF of patients with eosinophilic meningitis (EM).

Patient No.	B-SA for ES antigen	ES blocking test	B-SA for somatic antigen	somatic blocking test
8	-	ND*	+	-
9	+	-	ND	ND
10	-	-	-	ND
11	-	-	ND	ND

*not determined

Table 10 Assay for *Gnathostoma* ES and somatic antigens in CSF of patients with unspecified meningitis (UM).

Patient No.	B-SA for ES antigen	ES blocking test	B-SA for somatic antigen	somatic blocking test
12	-	ND	ND	ND
13	-	ND	ND	ND
14	+	-	ND	ND

*not determined

Table 11 Assay for *Gnathostoma* ES and somatic antigens in CSF of patients with other CNS involvements.

Patient No.	B-SA for ES antigen	ES blocking test	B-SA for somatic antigen	somatic blocking test
15	-	ND*	ND	ND
16	-	ND	ND	ND
17	-	ND	ND	ND
18	-	ND	ND	ND
19	-	ND	ND	ND
20	-	ND	ND	ND
21	-	ND	ND	ND
22	-	ND	ND	ND

*not determined

Table 12 Assay for *Gnathostoma* ES and somatic antigens in CSF of patients with unspecified illnesses.

Patient No.	B-SA for ES antigen	ES blocking test	B-SA for somatic antigen	somatic blocking test
29	-	ND*	ND	ND
30	+	-	+	-
31	-	ND	ND	ND
32	+	-	-	ND

*not determined

specific IgG antibody to L_3G somatic antigen. Moreover, these values were also used for the calculation of the following indices; specific activity (specific IgG titer to L_3G somatic antigen/total IgG), IgG-albumin index (CSF IgG/serum IgG ratio)/(CSF albumin/serum albumin ratio) and albumin ratio (serum albumin/CSF albumin). The six groups of patients used for these assays including

Group I: patient numbers 1-5 with CNS-G.

Group II: patient numbers 6 and 7 with SAH.

Group III: patient numbers 8-11 with EM.

Group IV: patient numbers 12-14 with UM.

Group V: patient numbers 15-28 with other CNS involvements.

Group VI: patient numbers 29-32 with unspecified illnesses.

3.2.1 Quantitation of specific IgG to L_3G somatic antigen

Specific IgG to somatic antigen in paired serum and CSF samples of these patients were assayed by ELISA as described in the Materials and Methods. The standard reference, both positive and negative controls, were included in every plate to allow adjustment of results obtained in analysis performing on different plates on different occasions. A typical titration curve for such a determination is shown in Figure 25. The end-point was arbitrarily taken to be the mid-point of the absorbance reading given by the serum dilution that approached the background value and that given by the dilution next to it (49). For the curve shown in Figure 25, the absorbance end point was found to be 0.22. The latter could then be transformed into the antibody titer corrected on the Y axis, which in this case was equivalent to an antibody titer of 1:150,000. This

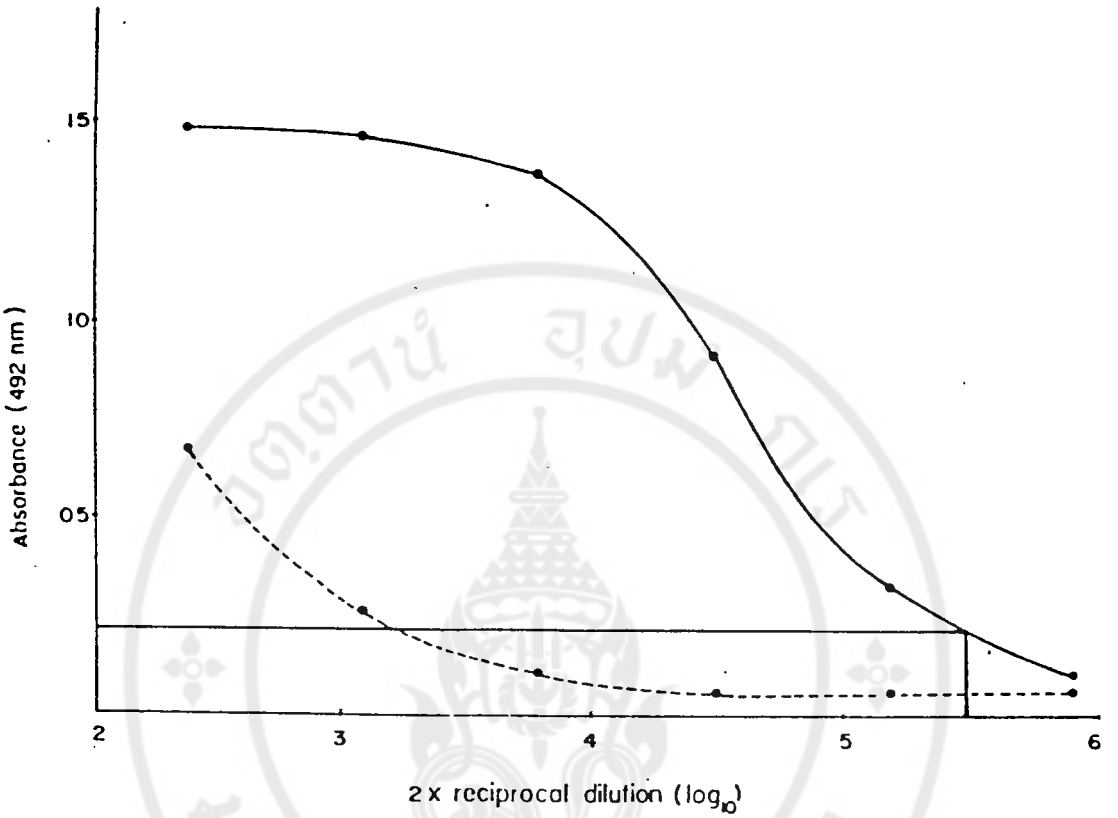


Figure 25 Standard titration curves for IgG antibody to L_3G in pooled positive (o——o) and negative (o---o) sera. Plates were coated with 50 µl of L_3G antigen at a protein concentration of 5 µg/ml. Sera were diluted 5-fold from 1:125 to 1:390,625, and each was incubated at 30°C for 60 min. The conjugated dilution at 1:900 was also incubated at 30°C for 60 min. Substrate was then added and the enzymatic reaction was allowed to take place in the dark at room temperature for 40 min.

pooled serum sample was subsequently included in all determinations to correct for a day-to-day variation.

The results presented in Figures 26 and 27 indicated the presence of specific IgG antibodies to somatic antigen in the serum and CSF of all patients with CNS-G and EM. Because the sample sizes of each group were small, non-parametic Kruskal-Wallis test was used to analyse the data. When comparing with values obtained from patients with other CNS involvements which in our case served as a control group, both CNS-G and EM groups exhibited significantly higher antibody titers ($p < 0.05$). However, there was no significantly different ($p > 0.05$) between the CNS-G and EM patients. The values obtained with patients having unspecified type of meningitis were within the normal range and were significantly different ($p < 0.05$) from those of the EM group (Table 13).

Both serum and CSF specimens from one of the two patients with SAH exhibited moderately high IgG antibody titer to L_3G somatic antigen (Figures 26 and 27). On the other hand, the other SAH patient had no detectable antibody in either specimens. In fact, the values obtained from this patient were within the normal range. However,it should be mentioned that this same patient (Patient No. 6 in Table 8) had ES antigen detectable in his CSF, suggesting the presence of active *G. spinigerum* infection.

With regarding to the presence of specific IgG antibody in either serum or CSF, the data clearly demonstrated a marked difference in patients with CNS-G and EM from those with other CNS involvements, including those with meningitis of unidentified etiology. Although no definite conclusion could be made from the

Table 13 Specific IgG antibody titers against L_3G somatic antigen in the serum and CSF specimens of patients with various CNS disorders.

Group	No. of cases	Antibody titer (log_{10}) $\bar{x}$ + SE	
		serum	CSF
Other CNS involvements (Negative control)	14	2.64+0.07	<0.69
Suggestive cerebral gnathostomiasis (CNS-G)	5	5.18+0.22*	3.4+0.46*
Eosinophilic meningitis presumably due to gnathostomiasis (EM)	4	4.17+0.32*	2.67+0.5*
Unspecified meningitis (UM)	3	2.6+0.25+	<0.69+

*Significantly different from the corresponding value of the control group ($p < 0.05$) by Kruskal-Wallis test.

+Significantly different from the EM group ($p < 0.05$) by Kruskal-Wallis test.

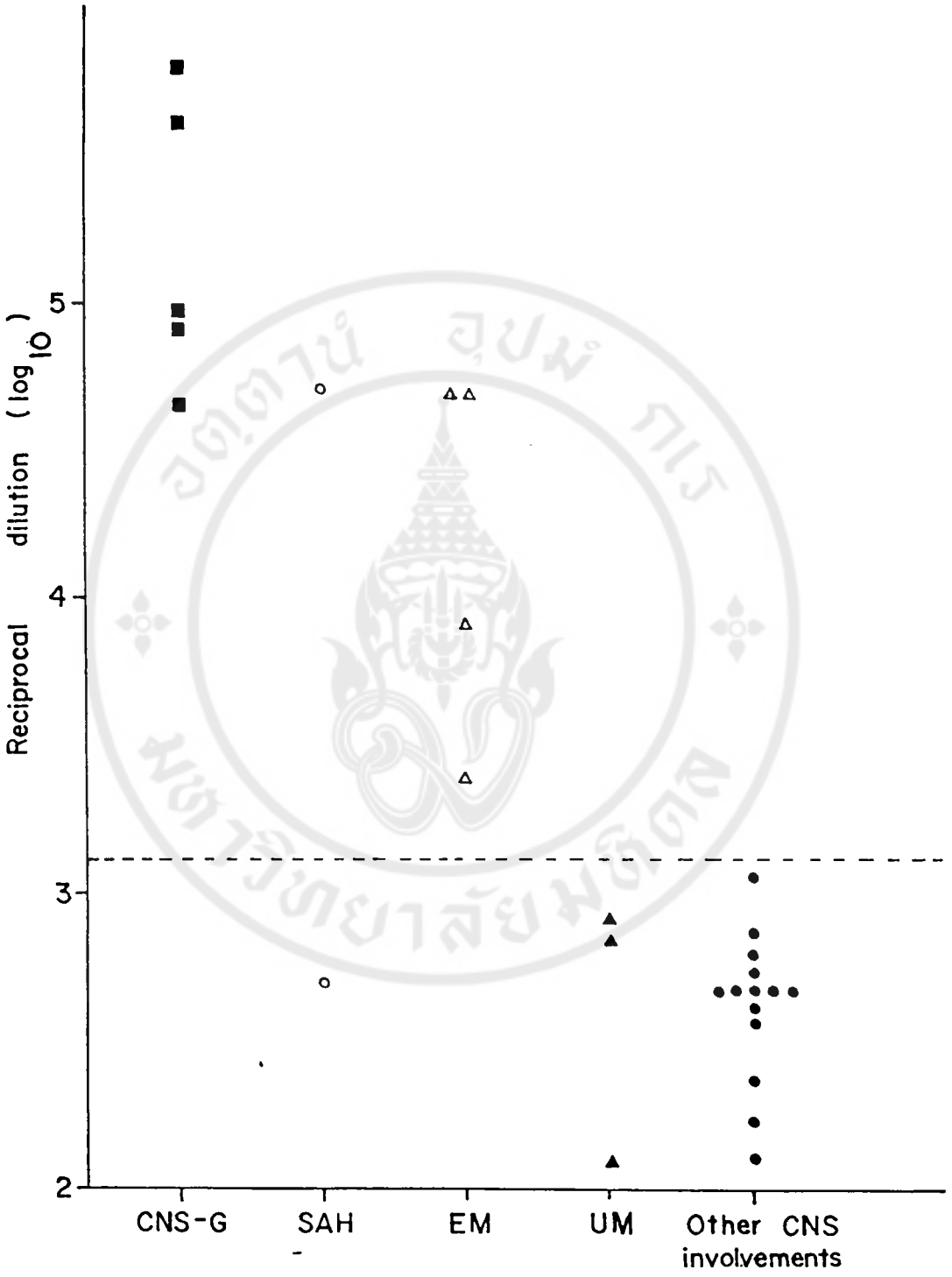


Figure 26 Distribution of specific IgG antibody titers against L_3G somatic antigen in the serum of patients with various CNS disorders. The dashed line indicated the $\bar{x}$ + 2SD of the control group (patients marked as other CNS involvements).

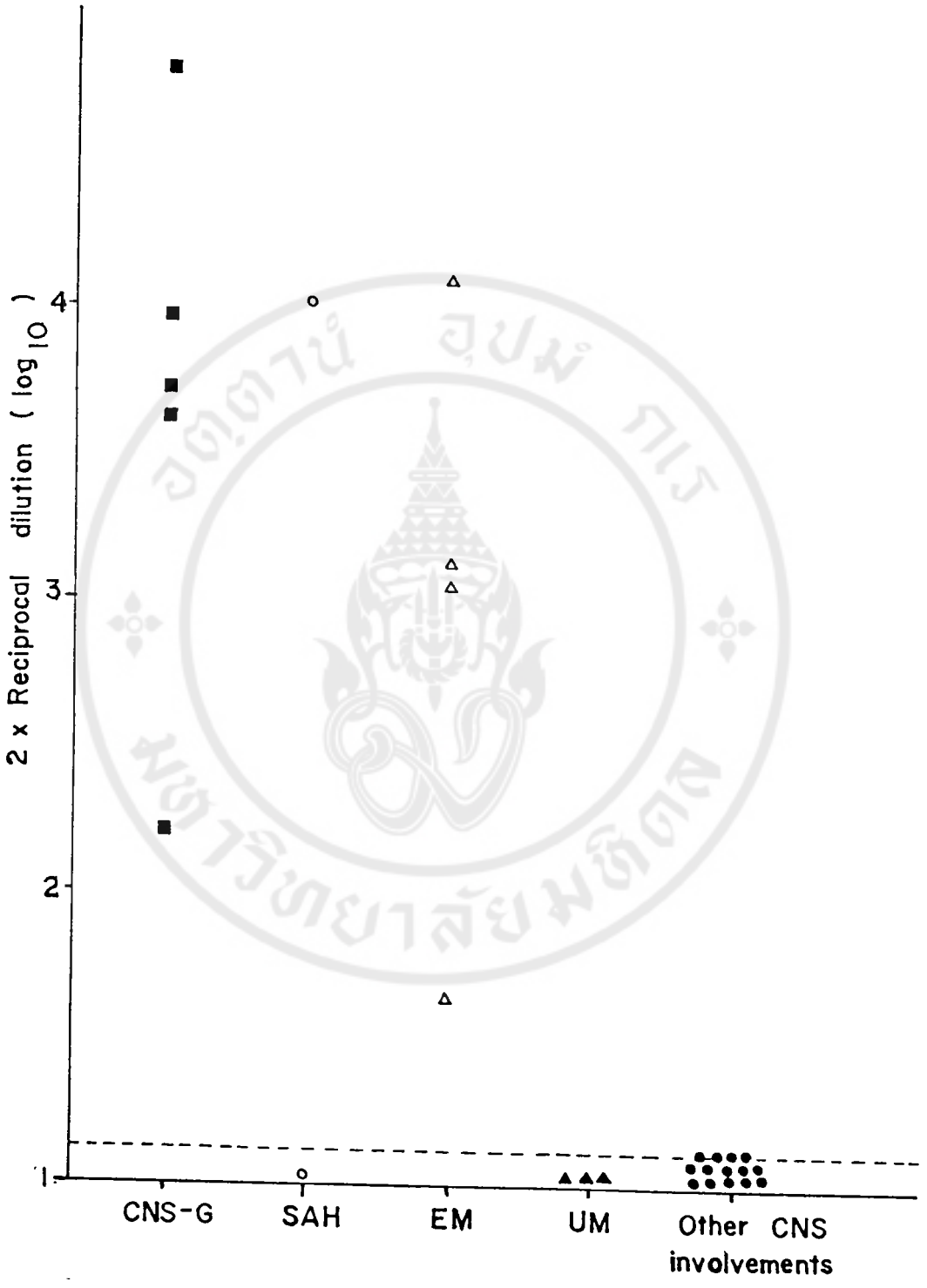


Figure 27 Distribution of specific IgG antibody titers against L_3G somatic antigen in the CSF of patients with various CNS disorders. The dashed line indicated the $\bar{x}$ + 2SD of control group (patients marked as other CNS involvements).

only 2 patients with SAH reported in this series of study, additional data available from a few more patients with SAH (not included in this study, data from R. Chawengkirttikul) showing elevated antibody titers in both serum and CSF specimens are consistent with the conclusion that gnathostomiasis may be responsible for the SAH in these patients. Unlike the specimens from these groups of patients, those from patients with unspecified illnesses gave variable results. For example, of the 4 patients in this group, only 2 patients exhibited significant elevation of serum antibody and of these 2 patients, only one had a significant elevation of antibody in the CSF specimen.

3.2.2 Quantitation of total IgG and albumin

In this section, total IgG and albumin in both the serum and CSF specimens were quantitated and the data obtained were used in conjunction with the antibody titers in the previous section, for the calculation of various indices and ratios, i.e., albumin ratio, IgG-albumin index and specific activities. Elevated IgG-albumin index together with high CSF specific activity would support the notion of local antibody synthesis within the CNS of these patients, particularly in those expressing normal serum/CSF albumin ratios. On the other hand, a depressed albumin ratio (i.e., increased CSF albumin) would suggest gross blood contamination. The latter may be associated with a procedure of lumbar puncture or with pathophysiological mechanism of the diseases itself, thus resulting in a damage of blood-brain barrier. These various parameters in patients suspected of having been infected with *G spinigerum* should

then be compared with those without neurological involvements reported by different groups of investigators (Table 14).

Data summerized as shown in Table 15 clearly demonstrated that IgG antibody could be detected in the CSF of all patients with cerebral gnathostomiasis. With the exception of patient no. 4, all other patients had moderate to high levels of antibody in the CSF specimens. However, analysis of serum/CSF albumin ratio for the possibility of gross blood contamination showed that the latter might have occured in patients no. 1 and 5. These 2 patients had very low serum/CSF albumin ratio and marked elevation of CSF albumin when compared with the normal value shown in Table 14. A corresponding albumin ratio for patient no. 3 also suggested that slight blood contamination could have been responsible for the presence of antibody in CSF of this patient.

If one accept that there was no gross blood contamination occurred in patients no. 2, 3 and 4, then the antibody detected in the CSF might have been due to local synthesis by antibody producing cells within the CNS or due to enhanced permeability of the vascular system in these patients. However, analysis of specific antibody activities in serum and CSF and the IgG-albumin index from these 3 patients suggested that only patient no. 2 may have local synthesis of antibody to *Gnathostoma* antigen and thus the elevated antibody levels in the other 2 patients (no. 3 and 4) may have been associated with enhanced permeability of serum antibody into the CNS.

Only one of the 2 patients (patient no. 7) in the SAH group had antibody demonstrable in both serum and CSF sample

(Table 16). Analysis of both the CSF albumin and serum/CSF albumin ratio in this patient failed to detect any gross blood contamination. Moreover, when specific antibody activities in CSF and serum were compared and together with IgG-albumin index, it appeared that the antibody detected in the CSF specimens was most likely synthesized locally within the CNS. Patient no. 6, on the other hand, had no antibody detectable in either the serum or CSF specimens, thus suggesting that the hemorrhage that occurred in this patient may have been caused by other factor(s). However, when the CSF was analysed for the presence of *Gnathostoma* antigens, a confirmed positive finding was noted (Table 8). Thus, this patient was also infected by *G. spinigerum*. The inability to detect free antibody may have been due to the presence of antibody in the form of immune complexes.

Like the other 2 groups of patients shown in Tables 15 and 16, the patients with eosinophilic meningitis all showed elevated antibody in CSF (Table 17). However, because the levels of CSF albumin were significantly elevated and, therefore, together with these depressed serum/CSF ratio in these patients suggested these antibodies may have been associated with gross blood contamination. On the other hand, the values of specific activity of CSF antibody and IgG-albumin index in patients no. 10 and 11 suggested also that these could have been some local synthesis of antibody. The findings in this group of patients were in marked contrast from those with meningitis associted with other causes (Table 18). There was no antibody to *Gnathostoma* antibody detectable in either the serum or CSF specimens. Similarly, both serum and CSF from all

Table 14 Reference immunological parameters in serum and CSF of patients without any known neurological involvements.

	Total IgG		Albumin		Albumin ratio (Serum/CSF)	IgG-albumin index
	Serum (mg/ml)	CSF (µg/ml)	Serum (mg/ml)	CSF (µg/ml)		
Tibbling et al., (50)	9.9-13.3[1]	16.0-32.0[1]	42.0-47.4[1]	141-261[1]	181.6-285.7	0.41-0.51
Olsson et al., (51)	7.2-11.0[2]	13.4-35.0[2]	36.2-50.4[2]	150-260[2]	193.8-241.0	0.36-0.56
Tungkanak et al., (52)	8.8-19.0[1]	23.0-41.0[2]	31.6-40.0[1]	97-181[1]	ND*	ND
Ramathibodi hospital	7.7-11.3[1]	16.0-32.0[2]	42.0-52.0[3]	ND	ND	ND

*not determined

[1]By radial immunodiffusion technique

[2]By electroimmunodiffusion technique

[3]By colorimetric method

Table 15 Specific activity of IgG to somatic antigen, albumin ratio and IgG-albumin index of patients with CNS-G.

Patient no.	Reciprocal dilution of specific IgG titers		Total IgG		Specific activities+		Albumin		Albumin# ratio (serum/CSF)	IgG-albumin index$
	serum	CSF	serum (mg/ml)	CSF (µg/ml)	serum	CSF	serum (mg/ml)	CSF (µg/ml)		
1	81,290	2,000	7.8	111.0	10.4	18.2	37.0	1,040.0	35.6	0.54
2	84,830	4,700	16.9	225.0	5.0	21.0	35.0	160.0	218.7	2.90*
3	420,000	2,500	13.8	57.5	30.4	43.0	27.0	230.0	117.4	0.49
4	46,250	83	8.6	16.5	5.4	5.0	31.5	138.0	228.3	0.44
5	650,000	30,000	15.0	500.0	43.3	60.0	41.0	1,580.0	25.9	0.86

*Suggestive IgG synthesis within CNS

+Specific IgG titers to L_3G somatic antigen/Total IgG

#Albumin in serum/Albumin in CSF

$(CSF IgG/serum IgG ratio)/(CSF albumin/serum albumin ratio)

Table 16 Specific activity of IgG antibody, albumin ratio and IgG-albumin index of patients with SAH.

Patient no.	Reciprocal dilution of specific IgG titers		Total IgG		Specific activities[+]		Albumin		Albumin[#] ratio (serum/CSF)	IgG-albumin index[$]
	serum	CSF	serum (mg/ml)	CSF (µg/ml)	serum	CSF	serum (mg/ml)	CSF (µg/ml)		
6	500	< 5	8.8	110.0	0.06	< 0.04	27.6	395.0	69.9	0.87
7	50,000	5,000	7.5	65.0	6.70	77.00	50.0	144.0	347.2	3.00*

*Suggestive IgG synthesis within CNS.

[+]Specific IgG titers to L_3G somatic antigen/Total IgG

[#]Albumin in serum/Albumin in CSF

[$](CSF IgG/serum IgG ratio)/(CSF albumin/serum albumin ratio)

Table 17 Specific activity of IgG antibody, albumin ratio and IgG-albumin index of patients with EM.

Patient no.	Reciprocal dilution of specific IgG titers		Total IgG		Specific activities+		Albumin		Albumin# ratio (serum/CSF)	IgG-albumin index$
	serum	CSF	serum (mg/ml)	CSF (µg/ml)	serum	CSF	serum (mg/ml)	CSF (µg/ml)		
8	50,776	577	10.0	65.0	5.1	8.90	33.6	400.0	84.0	0.55
9	2,400	22	8.1	75.0	0.3	0.29	52.0	605.0	85.9	0.79
10	50,000	6,000	13.1	135.0	3.8	44.40*	38.0	1,135.0	33.9	0.35
11	8,250	645	11.9	362.0	0.7	1.78	50.8	950.0	53.5	1.60*

*Suggestive IgG synthesis within CNS and contaminated serum in CSF.

+Specific IgG titers to L_3G somatic antigen/Total IgG

#Albumin in serum/Albumin in CSF

$(CSF IgG/serum IgG ratio)/(CSF albumin/serum albumin ratio)

Table 18 Specific activity of IgG antibody, albumin ratio and IgG-albumin index of patients with UM.

Patient no.	Reciprocal dilution of specific IgG titers		Total IgG		Specific activities+		Albumin		Albumin# ratio (serum/CSF)	IgG-albumin index$
	serum	CSF	serum (mg/ml)	CSF (µg/ml)	serum	CSF	serum (mg/ml)	CSF (µg/ml)		
12[1]	825	5	11.9	110.0	0.07	0.04	31.0	555.0	55.8	0.50
13[1]	<125	<5	8.8	47.5	<0.01	<0.10	29.0	313.0	92.6	0.50
14[2]	700	<5	11.9	262.5	< 0.60	<0.02	38.5	1,200.0	32.1	0.71

[1] Tuberculosis meningitis

[2] Subacute meningitis

*Suggestive IgG synthesis within CNS and contaminated serum in CSF.

+Specific IgG titers to L_3G somatic antigen/Total IgG

#Albumin in serum/Albumin in CSF

$(CSF IgG/serum IgG ratio)/(CSF albumin/serum albumin ratio)

Table 19 Specific activity of IgG antibody, albumin ratio and IgG-albumin index of patients with the other CNS involvements.

Patient no.	Reciprocal dilution of specific IgG titers		Total IgG		Specific activities+		Albumin		Albumin# ratio (serum/CSF)	IgG-albumin index$
	serum	CSF	serum (mg/ml)	CSF (µg/ml)	serum	CSF	serum (mg/ml)	CSF (µg/ml)		
15	700	< 5	15.0	20.0	0.05	<0.25	39.0	240.0	162.5	0.57
16	380	< 5	7.5	20.0	0.05	<0.25	43.0	140.0	307.1	0.82
17	1,300	< 5	11.9	29.0	0.11	<0.17	40.0	175.0	228.6	0.56
18	125	< 5	6.9	20.0	0.22	<0.25	38.6	330.0	116.9	0.34
19	850	< 5	13.8	30.0	0.06	<0.16	56.0	275.0	203.6	0.44
20	470	< 5	6.9	7.0	0.07	<0.70	56.0	31.0	1,806.4	1.87
21	240	< 5	6.9	65.0	0.03	<0.07	30.0	755.0	39.7	0.37

*Suggestive IgG synthesis within CNS.

+Specific IgG titers to L_3G somatic antigen/Total IgG

#Albumin in serum/Albumin in CSF

$(CSF IgG/serum IgG ratio)/(CSF albumin/serum albumin ratio)

Table 19 (Continued)

Patient no.	Reciprocal dilution of specific IgG titers		Total IgG		Specific activities+		Albumin		Albumin# ratio (serum/CSF)	IgG-albumin index$
	serum	CSF	serum (mg/ml)	CSF (µg/ml)	serum	CSF	serum (mg/ml)	CSF (µg/ml)		
22	170	< 5	8.8	7.5	0.02	< 0.67	37.0	70.0	528.6	0.45
23	525	6	7.5	65.0	0.07	0.09	46.0	800.0	57.5	0.49
24	450	< 5	6.9	8.5	0.06	< 0.58	43.0	148.0	290.0	0.36
25	500	< 5	10.0	47.5	0.05	< 0.10	54.0	750.0	72.0	0.34
26	625	< 5	11.9	40.0	0.05	< 0.12	50.0	310.0	161.3	0.54
27	488	< 5	6.9	47.5	0.07	< 0.10	50.0	125.0	400.0	2.70
28	550	< 5	7.5	65.0	0.07	< 0.07	48.0	875.0	54.8	0.47

*Suggestive IgG synthesis within CNS.

+Specific IgG titers to L_3G somatic antigen/Total IgG

#Albumin in serum/Albumin in CSF

$(CSF IgG/serum IgG ratio)/(CSF albumin/serum albumin ratio)

Table 20 Specific activity of IgG antibody, albumin ratio and IgG-albumin index of patients with unidentified illnesses involving CNS.

Patient no.	Reciprocal dilution of specific IgG titers		Total IgG		Specific activities[+]		Albumin		Albumin[#] ratio (serum/CSF)	IgG albumin index[$]
	serum	CSF	serum (mg/ml)	CSF (µg/ml)	serum	CSF	serum (mg/ml)	CSF (µg/ml)		
29	215	< 5	8.6	7.5	0.03	< 0.67	35.0	100.0	350.0	0.31
30	600,000	20,000	11.9	1,000.0	50.40	20.00	31.0	305.0	101.6	8.50[*]
31	< 125	< 5	5.4	65.0	< 0.03	< 0.07	27.0	391.0	69.0	0.83
32	3,700	< 5	10.0	27.0	0.06	< 0.04	35.0	76.0	460.0	1.20

[*] Suggestive IgG synthesis within CNS and contaminated serum in CSF.

[+] Specific IgG titers to L_3G somatic antigen/Total IgG

[#] Albumin in serum/Albumin in CSF

[$] (CSF IgG/serum IgG ratio)/(CSF albumin/serum albumin ratio)

patients with other CNS involvements had no antibody to *Gnathostoma* antigen.

Of the 4 patients shown in Table 20, only patient no. 30 had high levels of antibody to *G. spinigerum* in both serum and CSF specimens. Other parameters found in this one patient are consistent with local synthesis of antibody within the CNS.

4. Detection of immune complexes in cerebrospinal fluid

Because data obtained in the previous sections showed that with the exception of one patient (no. 6) free L_3G antigen could not be detected in CSF of patients suspected of having been infected with *G. spinigerum*, it is logical to suspect that these antigens may have been present in the form of immune complexes. This is particularly more obvious in view of the fact that a majority of these patients had high level of IgG antibody in the CSF. Therefore, an experiment was performed to detect the possible presence of immune complexes in these CSF specimens, using complement-consumption test. However, prior to being quantitated, the concentration of immune complexes that might be present only in trace quantity in the CSF of these patients had to be increased by PEG precipitation as described in the Materials and Methods section. The concentrated immune complexes in CSF were then assayed for their ability to activate complement in the test system and residual hemolytic complement activity in the system was assayed for CH_{50} by microtitration technique as previously described in section 11.2. Diagram presented in Figure 28 represents an example of such an assay. In this test, the NHS to be used as a source of hemolytic complement in the system

had to be titrated for complement activity, using two-fold dilution, before adding to the concentrated CSF samples. The CH_{50} or the serum dilution giving 50% hemolysis was 1:16. In this figure, after the concentrated CSF from the patient number 10 was added, the CH_{50} was reduced from 1:16 to 1:12, indicating a consumption of approximately 25% of complement in the test system. Details of such a determination in patients whose CSF was available in sufficient quantity are shown in Tables 21-26.

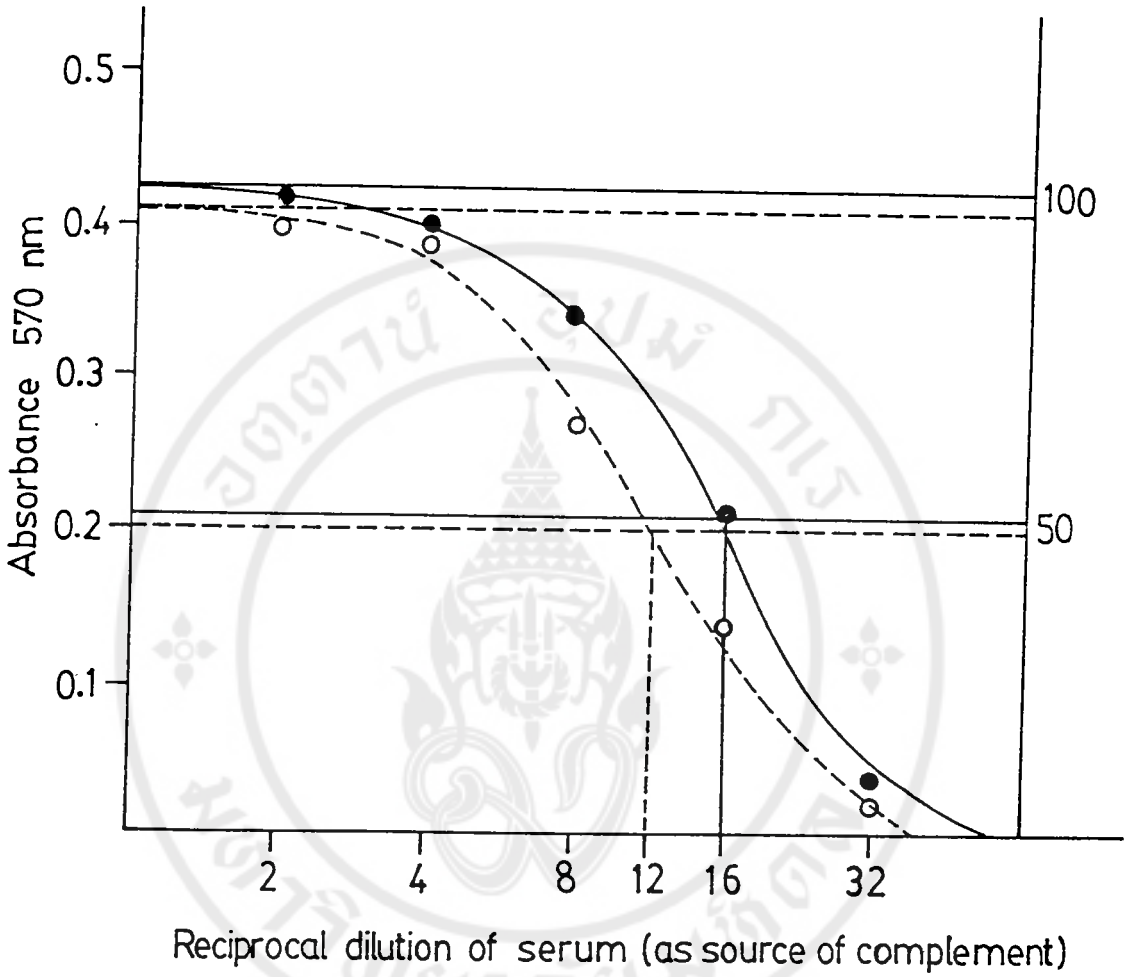


Figure 28 Dose response curve of complement - mediated lysis of sensitized sheep erythrocytes performed in the absence (o——o) or presence (o---o) of immune complexes. Using 50% hemolysis (CH_{50}) as the end point for this complement-consumption test, the presence of immune complexes in unknown specimen reduced the complement titer from 16 units to 12 units.

Table 21 Immune complexes in CSF of patients with CNS-G.

Patient No.	Reciprocal dilution of IgG-titers in CSF	Reciprocal dilution giving 50% lysis (CH_{50})	Complement consumption (%)	Immune complexes
1	2,000	16	0	-
2	4,700	16	0	-
3	2,500	16	0	-
4	83	ND*	ND	ND
5	30,000	16	0	-

*not determined.

Table 22 Immune complexes in CSF of patients with SAH.

Patient No.	Reciprocal dilution of IgG-titers in CSF	Reciprocal dilution giving 50% lysis (CH_{50})	Complement consumption (%)	Immune complexes
6	< 5	16	0	-
7	5,000	16	0	-

Table 23 Immune complexes in CSF of patients with EM.

Patient No.	Reciprocal dilution of IgG-titers in CSF	Reciprocal dilution giving 50% lysis (CH_{50})	Complement consumption (%)	Immune complexes
8	577	16	0	-
9	22	ND*	ND	ND
10	6,000	12	25	+
11	645	16	0	-

*not deterimined

Table 24 Immune complexes in CSF of patients with UM.

Patient No.	Reciprocal dilution of IgG-titers in CSF	Reciprocal dilution giving 50% lysis (CH_{50})	Complement consumption (%)	Immune complexes
12	5	16	0	-
13	< 5	ND*	ND	ND
14	< 5	16	0	-

*not determined

Table 25 Immune complexes in CSF of patients with other CNS involvements.

Patient No.	Reciprocal dilution of IgG-titers in CSF	Reciprocal dilution giving 50% lysis (CH_{50})	Complement consumption (%)	Immune complexes
15	< 5	ND*	ND	ND
16	< 5	16	0	-
17	< 5	16	0	-
18	< 5	ND	ND	ND
19	< 5	ND	ND	ND
20	< 5	16	0	-
21	< 5	16	0	-
22	< 5	ND	ND	ND
23	6	ND	ND	ND
24	< 5	ND	ND	ND
25	< 5	ND	ND	ND
26	< 5	ND	ND	ND
27	< 5	ND	ND	ND
28	< 5	ND	ND	ND

*not determined

Table 26 Immune complexes in CSF of patients with unidentified illnesses involving CNS.

Patient No.	Reciprocal dilution of IgG titers in CSF	Reciprocal dilution giving 50% lysis (CH_{50})	Complement consumption (%)	Immune complexes
29	< 5	16	0	-
30	20,000	16	0	-
31	< 5	ND*	ND	ND
32	< 5	16	0	-

*not determined

5. Characterization of L_3G anitigens.

5.1 Protein profiles by SDS-PAGE

The complexity of protein components in the various L_3G preparations were analysed by SDS-PAGE and stained with either Coomassie blue or silver staining. The examples of the SDS-PAGE patterns are shown in Figures 29 and 30 and the total number of polypeptides together with the range of molecular weights for each polypeptide are summerized in Table 27. The silver nitrate profiles of both somatic extract and ES antigen appeared to be considerably more complex than that of detergent extract. The range of molecular weights of somatic extract were broader ranging between 13 and more than 116 kilodaltons (KD) whereas those of the ES were between 12 and 98 KD. However, the protein pattern of the detergent-extract surface antigen exhibited a much more simple pattern (Figure 30) than either the somatic extract or ES antigen; with the silver staining there were 11 distinct bands of equal intensity with molecular weight ranging between 16 and 70 KD. All of these *Gnathostoma* preparations had polypeptide with molecular weight of 44 KD but the relative proportion of this component varied from one preparation to another.

The Coomassie blue staining pattern of the somatic extract showed a major band at the 38 and 39 KD position which coincided with the predominate band previously described by Priwan (16) and less intensely staining bands at 16, 20, 22, 36, 41, 44, and 49 KD positions. In addition, there were many other higher molecular components that were present in trace quantity. On the other hand, the predominant bands in the metabolic products had molecular weight

of 45 and 49 KD. Most of the remaining components had molecular weight between 40 and 44 KD. Only one or two bands were noted in the lower molecular weight range. Due to its availablity in limited quantity, the SDS-PAGE pattern of the surface extract could not be seen by the Coomassie blue staining.

5.2 Glycoprotein staining

To further characterize the biochemical properties of somatic and ES antigens of L_3G, these antigens were allowed to react with concanavalin-A, a lectin with specificity for mannose, glucose and N-acetylglucosamine residues (53), and the lectin was subsequently detected with horseradish peroxidase. Unlike the protein staining, the glycoprotein staining of the somatic extract was very simple, with only one faint band at the 38 KD position (Figure 31). From its position on the gel, this glycoprotein component appeared to be identical with the major protein component in this somatic extract (Table 28). Contrary to somatic antigen, the ES components with molecular weight 44, 45 and 49 KD were heavily glycosylated and the bands with molecular weight ranging from 33 to 39 KD and 53 to 62 KD were lightly glycosylated (Table 29).

5.3 Immunoenzymatic blotting of L_3G antigens with sera from gnathostomiasis patients

Electrophoresed L_3G components were transferred to a nitrocellulose membrane as described in the Materials and Methods. Under the condition used, the transfer was almost complete as verified by a similar protein staining pattern before and after the transfer (Figure 32 A and Figure 32 B, lanes 1 and 2). Additional evidence

to support the above conclusion comes from the intensity of protein staining remaining on the gel subsequent to the blotting procedure. The incomplete transfer of protein was noted only in the high molecular weight components, particularly with those having molecular weight more than 116 KD.

The ability of the blotted proteins to react with antibodies present in the various immune sera was detected by allowing them to react with a 1:3,000 dilution of pooled positive serum from patients with gnathostomiasis (Figure 33, lane 2). The most strongly reactive band was noted at a 44 KD position, with a lesser intensity at 33, 26, 21 and 19 KD. However, a lesser but detectable reactivity was also noted at several other positions ranging from 62 to > 116 KD. An identical pattern of reaction was noted with the serum from a patient with parasitologically prove gnathostomiasis (Figure 33, lane 3). In contrast, none of these components reacted with pooled normal human sera tested at the same dilution (Figure 33, lane 4).

Unlike the somatic antigen, when the ES antigen was used to react with the same pooled positive serum from patients with gnathostomiasis most reactivities were noted at the 21 to 49 KD positions as summerized in Table 29. The two most intensely stained components had molecular weight 44 and 49 KD (Figure 34, lane 3). A less intensely stained proteins were 53 to 65 and 13 to 20 KD. It should be noted that none of these immunogenic components gave a positive reaction with pooled normal human sera tested at the same dilution (Figure 34, lane 4).

5.4 Immunoenzymatic blotting of L_3G with hyperimmune rabbit sera

To characterize the L_3G antigens in more details, hyperimmune rabbit sera against the various L_3G antigens (somatic, ES, surface antigens) raised as described in Section 5 were also used in the immunoblotting. The dilution of these rabbit antisera were determined from their antibody titers. The optimum dilution was 1:3,000 for anti-somatic and 1:1,000 for both anti-ES and surface antigens. Because of the low protein concentration and the limited amount of surface extract currently available, only the somatic extract was used to react with anti-surface extract in this immunoblotting experiment. Results shown in Figure 35 (lanes 1 and 2) demonstrated that the 44 KD peptide reacted most strongly with both anti-somatic and anti-surface. In fact, this was the only significant component in the somatic extract that reacted with anti-surface extract. Anti-somatic gave a pattern of reaction similar to that reacted with the pooled gnathostomiasis sera (Figure 35, lane 4). The one difference was noted that the 19 KD component reacted with human gnathostomiasis sera but not reacted with rabbit hyperimmune sera. These results are summerized in Table 28.

Unlike rabbit anti-somatic serum and human gnathostomiasis serum, rabbit anti-ES reacted with only a limited number of antigens in the ES preparation. As shown in Figure 34, anti-ES reacted largely only with components having molecular weight between 23 and 65 KD positions. The distinctive difference from the pattern noted with human sera was the low molecular weight ranging between 13 and 21 KD. These results are summerized in Table 29.

5.5 Immunoenzymatic blotting of L_3G antigen with infected mouse sera

As mouse and human are accidental host for *G. spinigerum*, it is interesting to compare the immune response in these two species. For this experiment, sera from mice experimentally infected with 15 larvae were also used in immunoblotting. The pooled mouse sera with high ELISA titers (1:32,000) was used at a 1:1,000 dilution. Although the overall pattern observed with mouse serum was similar to that of human (comparing lane 3 with lane 4 in Figure 35), some differences were noted (Table 28), particularly in the high molecular weights. With both sera, the strongest reaction was noted at 44 KD position. Other components that reacted fairly strongly with both sera including those with molecular weight of 19 and 21 KD. It should be noted that many high molecular weight components only reacted intensely with mouse serum (46 KD to > 116 KD). Lower molecular weight components (16 KD to 25 KD) also reacted strongly with mouse serum but only faintly, if any, with human serum.

5.6 Immunoenzymatic blotting of L_3G with angiostrongyliasis serum

In order to determine species specific and common antigens of the L_3G, somatic and ES antigens of *Gnathostoma* were allowed to react with pooled positive serum from patients infected with *A. cantonensis* or with *G. spinigerum* and their immunoreactive patterns were compared. The one most distinctive pattern obtained with *A. cantonensis* sera is that it reacted strongly with high molecular weight components (44-116 KD) and very little if at all with low

molecular weight (16-33 KD), while it failed to react with ES antigen (Figure 32 B). It is of interest to note that the predominant band of *Gnathostoma* somatic antigen (39 KD) reacted poorly with gnathostomiasis serum but it is the component that reacted most strongly with *A. cantonensis* serum.

Table 27 The number of polypeptides and the estimated molecular weight of various L_3G antigens.

Antigen	Coomassie blue		Silver staining		Predominant major bands
	No. of band	MW (KD)	No. of band	MW (KD)	
somatic	28	>116-13	40	>116-13	38 and 39
ES	20	62-15	25	98-12	45 and 49
surface extract	-*	-	11	70-16	-**

* None of them stained by Coomassie blue.

** No distinctive major band.

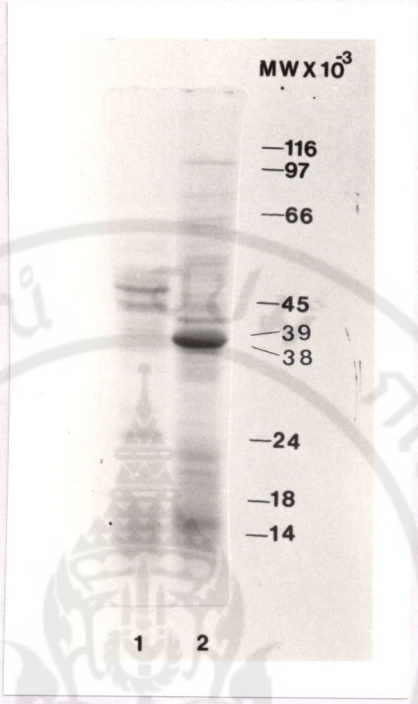


Figure 29 Coomassie blue stained SDS-PAGE profiles of L_3G ES antigen (lane 1) and somatic antigen (lane 2). The major bands of the ES antigens represent components with molecular weight (MW) of 45 and 49 KD, whereas those of the somatic antigen represent components with MW 38-39 KD. Molecular weight markers are shown on the right.

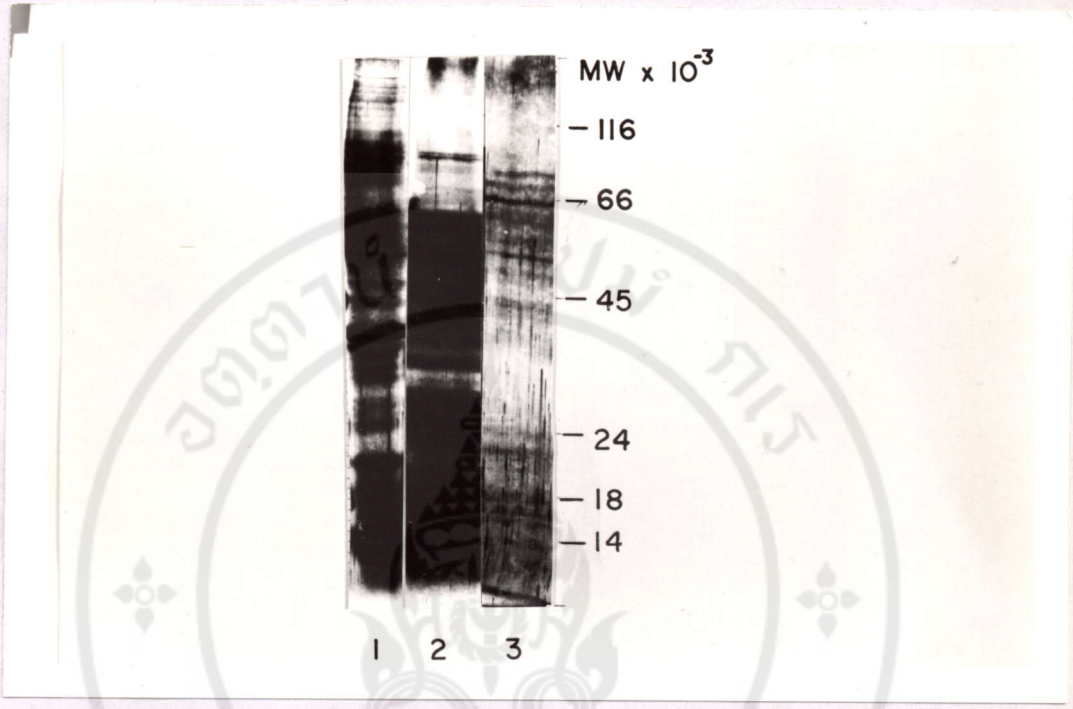


Figure 30 Silver stained SDS-PAGE profiles of somatic extract (lane 1), metabolic products (ES, lane 2) and surface extract (lane 3). Molecular weight (MW) markers are shown on the right.

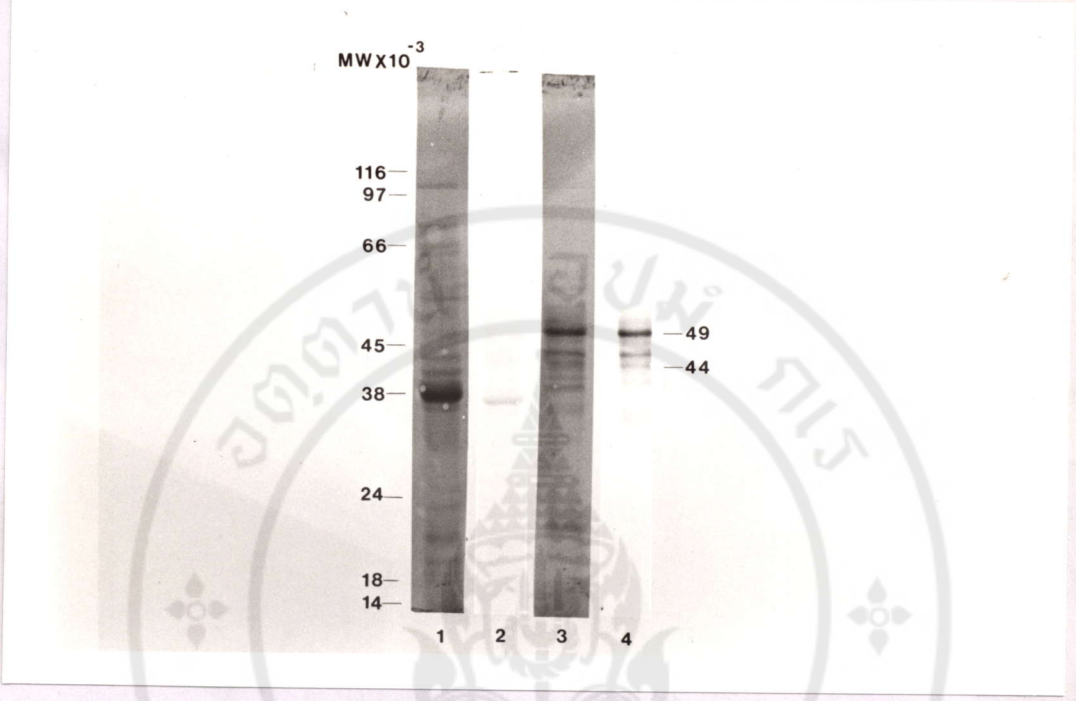


Figure 31 Electrophoretic blotting of L_3G Somatic antigen (lanes 1 and 2) and ES antigen (lanes 3 and 4) stained with Amido-black (lanes 1 and 3) and with concanavalin-A (lanes 2 and 4). Molecular weight markers are shown on the left.

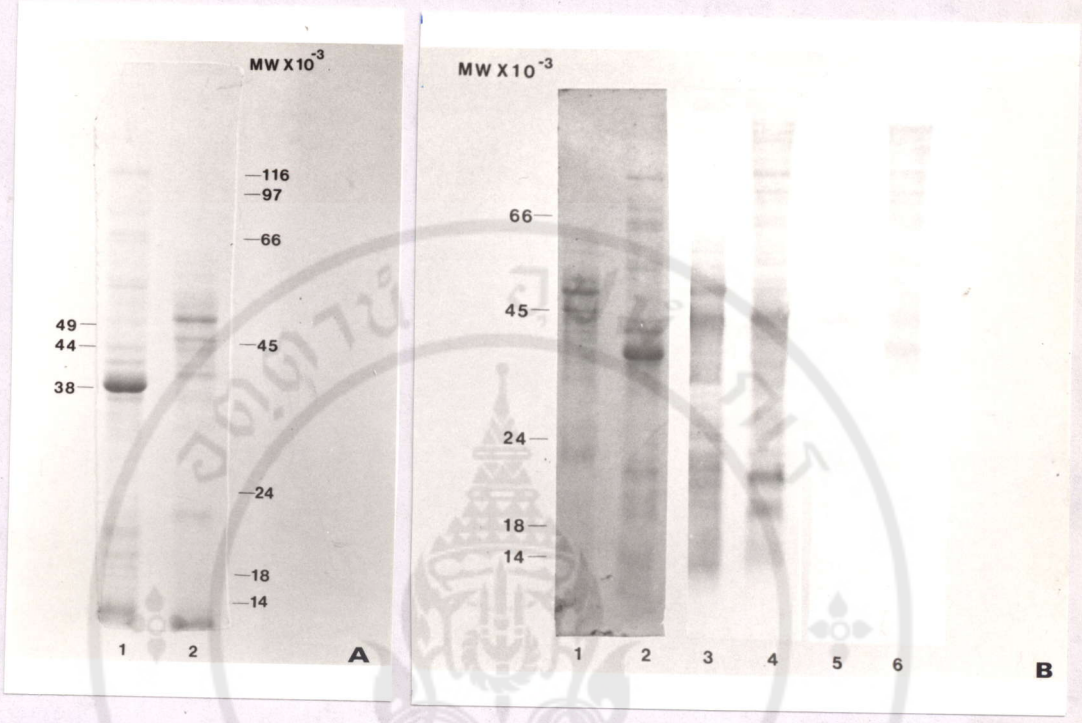


Figure 32 A Coomassie blue stained SDS-PAGE profiles of L_3G somatic antigen (lane 1) and ES antigen (lane 2)

B Electrophoretic blotting of ES antigen (lanes 1, 3 and 5) and somatic antigen (lanes 2, 4 and 6) from polyacrylamide gel to nitrocellulose and stained with Amido-black before (lanes 1 and 2) and after reacting with pooled positive gnathostomiasis serum (lanes 3 and 4) and pooled positive angiostrongyliasis serum (lanes 5 and 6). The antibodies reacting with these components were detected with goat anti-human IgG conjugated-alkaline phosphatase.

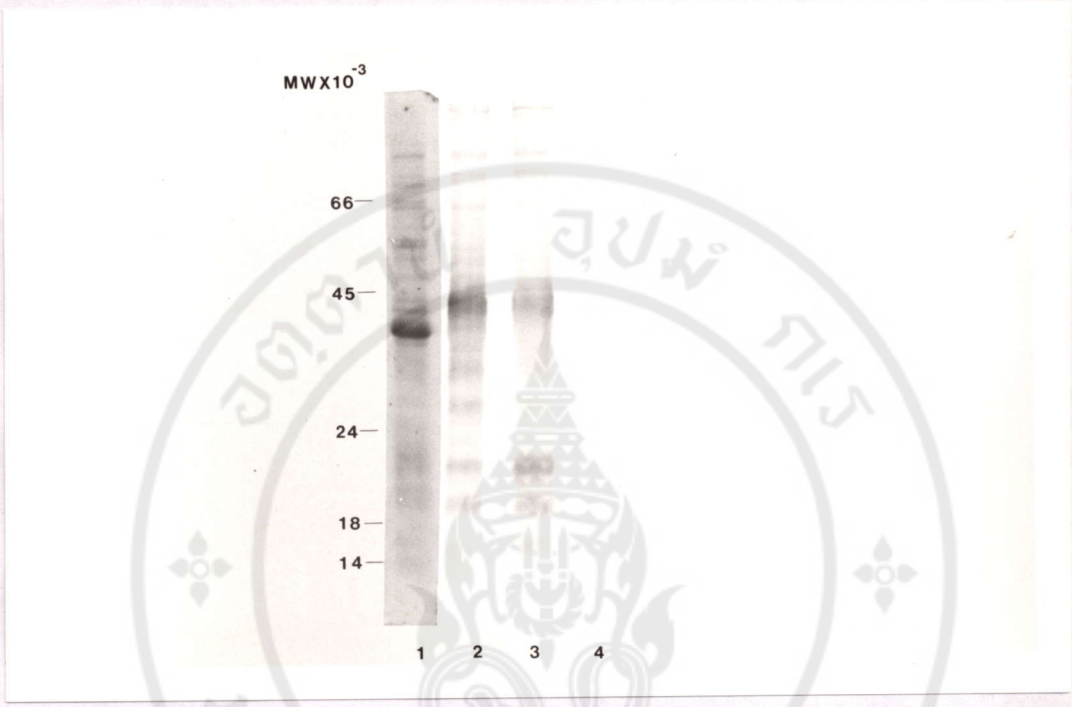


Figure 33 Electrophoretic blotting of L_3G somatic antigen from polyacrylamide gel to nitrocellulose and stained with Amido-black before (lane 1) and after reacting with pooled positive gnathostomiasis serum (lane 2), with parasitologically proven gnathostomiasis serum (lane 3) and with pooled normal human sera (lane 4). The antibodies reacting with these components were detected with goat anti-human IgG conjugated with alkaline phosphatase.

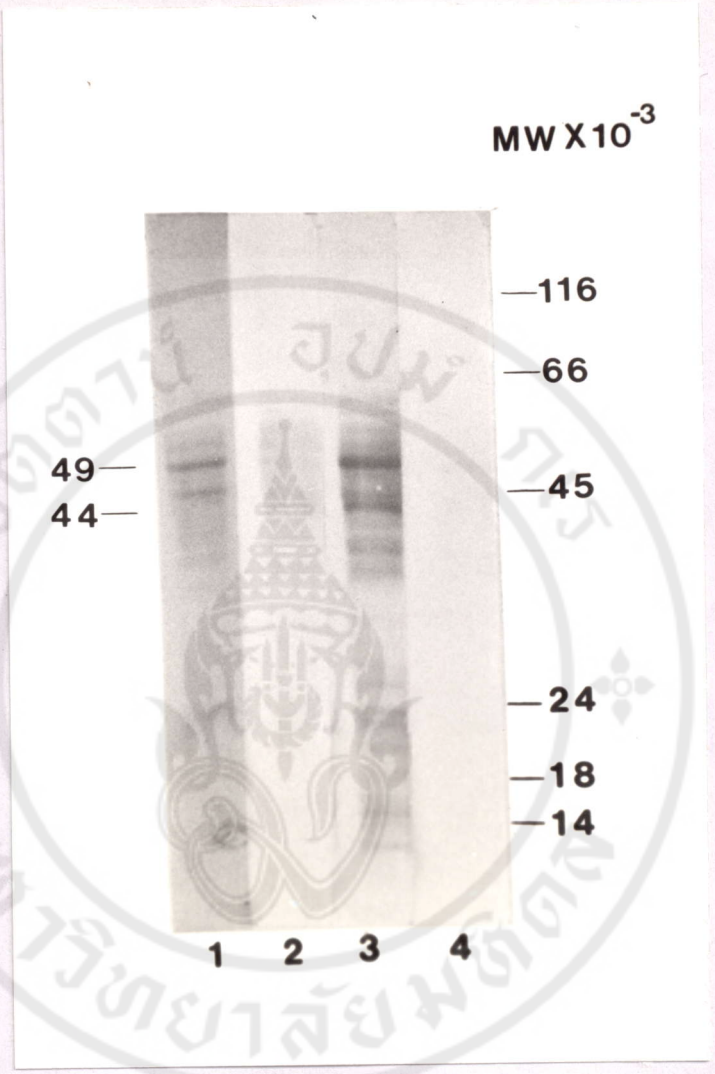


Figure 34 Electrophoretic blotting of ES antigen on nitrocellulose Amido-black stained protein pattern before (lane 1) and after reacting with rabbit hyperimmune serum against ES antigen (lane 2), with pooled positive gnathostomiasis serum (lane 3) and with pooled normal human serum (lane 4). The most reactive band remarked on the left.

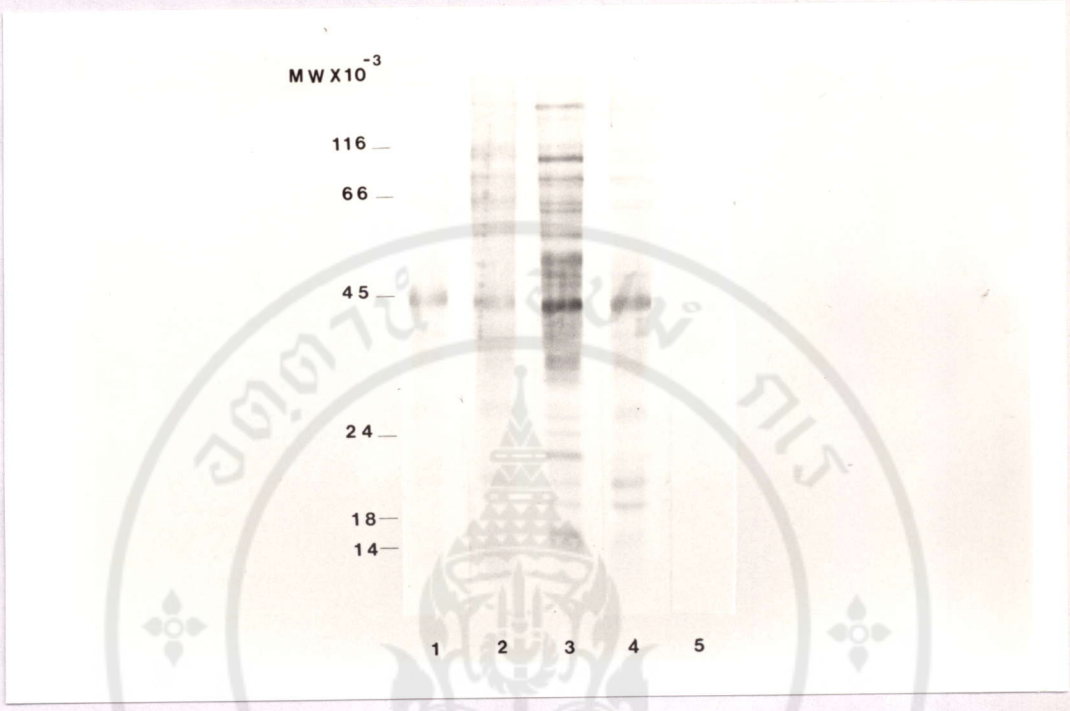


Figure 35 Immunoenzymatic blotting of somatic antigen with hyperimmune rabbit serum against surface extract (lane 1), and somatic antigen (lane 2) and with pooled sera from infected mice (lane 3), and humans (lane 4) and reacting with pooled normal rabbit serum was used as negative control (lane 5). The antibodies reacting with these components were detected with goat anti-rabbit IgG, rabbit anti-mouse immunoglobulin and goat anti-human IgG conjugated with alkaline phosphatase, respectively.

Table 28 Physicochemical and immunological properties of somatic antigen.

Band no.	MW (KD)	Protein profiles	Glycoprotein staining	Immunoenzymatic blotting in the presence of				
				Gnathostomiasis sera from				Angiostrongyliasis human sera
				human	mouse	rabbit anti-somatic	rabbit anti-surface	
1	>116	-*	-	±	4+	±	-	-
2	116	-	-	±	±	±	-	+
3	100	2	-	±	4+	±	-	+
4	70	+	-	±	+	±	±	+
5	68	+	-	±	+	±	±	+
6	64	2+	-	±	+	±	±	+
7	62	2+	-	±	+	±	±	-
8	55	±	-	-	2+	-	-	-
9	53	±	-	-	4+	-	-	-
10	49	±	-	+	+	-	-	-
11	46	±	-	-	+	-	-	-
12	44	2+	-	4+	4+	4+	4+	2+

Table 28 (Continued)

Band no.	MW (KD)	Protein profiles	Glycoprotein staining	Immunoenzymatic blotting in the presence of				
				Gnathostomiasis sera from				Angiostrongyliasis human sera
				human	mouse	rabbit anti-somatic	rabbit anti-surface	
13	39	4+	-	-	-	-	-	4+
14	38	4+	+	-	-	-	-	-
15	33	±	-	+	3+	±	±	-
16	32	±	-	-	+	-	-	-
17	26	±	-	2+	+	+	±	-
18	25	±	-	±	3+	±	-	-
19	23	-	-	±	4+	-	-	-
20	21	+	-	4+	2+	2+	±	-
21	19	±	-	4+	2+	-	-	-
22	16	±	-	±	4+	±	-	-

* Intensity of the protein band (± to 4+).

Table 29 Physicochemical and immunological properties of ES antigen.

Band no.	MW (KD)	Protein profiles	Glycoprotein staining	Immunoenzymatic blotting in the presence of		
				Gnathostomiasis human sera	sera from rabbit anti-ES	Angiostrongyliasis human sera
1	65	±*	-	+	+	-
2	62	±	+	+	+	-
3	61	±	+	+	+	-
4	56	±	2+	+	+	-
5	53	±	2+	+	+	-
6	49	4+	4+	4+	4+	±
7	45	4+	4+	-	-	-
8	44	3+	3+	4+	4+	±
9	39	+	+	2+	±	±
10	38	±	±	2+	±	-
11	33	±	±	2+	±	-
12	27	±	-	+	±	-

Table 29 (Continued)

Band no.	MW (KD)	Protein profiles	Glycoprotein staining	Immunoenzymatic blotting in the presence of		
				Gnathostomiasis human sera	sera from rabbit anti-ES	Angiostrongyliasis human sera
13	25	±	-	+	±	-
14	23	3+	-	2+	±	-
15	21	±	-	2+	-	-
16	20	±	-	+	-	-
17	18.5	-	-	+	-	-
18	17	-	-	+	-	-
19	16	-	-	+	-	-
20	15	-	-	+	-	-
21	13	-	-	+	-	-

* Intensity of the protein band (± to 4+).

CHAPTER V

Discussion

Results presented in this study have given detailed information regarding the *Gnathostoma* antigens, particularly those with potential in immunodiagnosis of gnathostomiasis. Moreover, the data allow one to more critically analyze antibody in the CSF of patients with cerebral gnathostomiasis, thus giving further information on the possible origin of these antibodies and their potential in the diaghosis of cerebral gnathostomiasis. Regarding the latter aspect, it is possible to conclude that local synthesis of antibody does occur within the CNS of some patients with cerebral gnathostomiasis and that when the CNS is involved, the CSF can be used for the detection of antibodies.

Different lines of evidence presented in this study suggest that both the metabolic products and somatic extract of third-stage *Gnathostoma* larvae could be used in the serodiagnosis of gnathostomiasis. In fact, the data obtained suggest that the metabolic products may be a better choice and, therefore, detailed studies of this material was undertaken and compared with the somatic extract.

Although with the *in vitro* condition used in this study, it was possible to maintain L_3G metabolically active for more than 2 weeks, the production of ES in this study was terminated and the spent medium was collected within 4 days of incubation because it was found that during this period of incubation most activity, as monitored by its protein content, was detected (Figure 1). One additional advantage of terminating the ES production during this short incubation period is to minimize contamination with somatic

components released by dying worms which began at around day 7 onward. It was thus calculated that under the *in vitro* condition used in this study, during the peak period within 4 days of incubation, each larva secreted approximately 700 ng of protein content per day. Such a minute amount of ES produced and secreted by these larvae may help explaining why we could not demonstrate the presence of ES antigen in the CSF in a majority of patients with cerebral gnathostomiasis. With other parasitic infection, antigens could be detected in some body fluids and serum of infected animals. In these situations, each person may be infected by a large number of parasites, thus producing sufficient quantity of antigens for quantitation. On the other hand, some parasites can multiply in their definitive hosts and can increase their number within a brief interval, thus making enough antigens for detection. *G. spinigerum*, however, is an accidentally infected parasite which cannot reproduce in humans and it is believed that each person is infected by only one infective larva (5, 54). Such a phenomenon reduces a chance of detecting antigens, even when one confines to a small circumscribed area within CNS. However, a detection of parasite antigen has never been reported in CSF.

When the protein profile of concentrated ES products was compared with those of somatic and surface extract of L_3G (Figures. 29-30, Table 27), strikingly different protein and glycoprotein staining patterns were noted. Regarding the somatic extract, the protein pattern noted in the present study was similar to those reported previously by Priwan (16) and Nopparatana *et al.*, (27). In all of these studies, a wide range of different molecular weight

components from more than 116 to 13 KD was observed. With all 3 studies, the predominant protein component in the somatic extract had molecular weight around 38 KD. In this study, additional data demonstrated that this component was a glycoprotein and it was the only major glycoprotein component present in the somatic extract. Although both Priwan (16) and Nopparatana *et al.*, (27) reported previously that this 38 KD major component reacted with both human and mouse serum obtained from those infected with *G. spinigerum*, in this study, it reacted poorly with gnathostomiasis serum (Figure 32 B, lane 4). In fact, the reactivity was much stronger with the other components e.g., 44, 26, 21 and 19 KD. However, this major component reacted more intensely with serum from angiostrongyliasis patients, but cross-reactivity with other parasites has not yet been tested. Priwan (16) found that this major component adsorbed strongly with protein-A Sepharose beads used as a carrier in her system. Likewise, Nopparatana *et al.*, (27) also used I^{125} labelled protein-A to detect the presence of antigen-antibody complexes blotted onto a nitrocellulose sheet and found that this 38 KD component reacted weakly with all sera from healthy persons. These results suggested either the presence of a truly cross reactive antibodies in the serum of normal individuals or the presence of a technical artifact, i.e., a non-specific binding to protein-A Sepharose. The latter possibility can be excluded by a preabsorption of the antigen with protein-A Sepharose prior to being used in the system or used antigen to react directly with protein-A Sepharose.

The protein profile of the ES products obtained from an *in vitro* maintenance of L_3G in protein-free medium in the present study was markedly different from that of its somatic counterpart. Such a difference in protein patterns have been noted with other nematodes (55-57). On the other hand, Maleewong *et al.*, (58) recently found that the ES antigens used in their study for the detection of specific antibody in gnathostomiasis serum by ELISA gave identical results with those obtained with somatic antigens, thus giving a circumstantial evidence for a similarity between these two antigen preparations. The discrepancy between these 2 studies could be readily explained by the difference of culture conditions and/or the optimal time for collecting the ES component. In the study reported herein, all precantions were undertaken to minimize any possible contamination of ES products by any somatic component, and both the protein and glycoprotein patterns shown in Figure 31 strongly supported this contention. For instance, when the gel was stained with Coomassie blue, the ES preparation obtained failed to show the presence of any protein in high molecular weight region that were abundantly present in the somatic extract (Figure 29). Moreover, the 38 KD major somatic component was present only in trace quantity in the ES. In the latter, a majority of the proteins has molecular weight between 55 to 40 KD and it was interesting to note that practically all proteins in this region reacted intensely with concanavalin-A (Figure 31). Thus, in contrast to the somatic components which were almost entirely protein in nature, practically all components in the ES preparation were glycoprotein.

In order to identify and characterize which of these various components are good immunogens with a potential for diagnosis of human gnathostomiasis, the various components of L_3G were used in the immunoblot analysis with human sera from gnathostomiasis and angiostrongyliasis. In the immunoblot analysis, these various proteins and glycoproteins were found to be highly immunogenic as they reacted strongly with the sera from infected humans and mice and also from rabbits immunized with various antigen preparations (see Figures 34-35). The immunoblot patterns of somatic extract obtained after reacting with antisera from rabbits immunized with this antigen showed a highly complicated pattern. The results presented in Figure 35 showed that practically all protein components in the somatic preparation were highly immunogenic except the major 38-39 KD components which reacted poorly with the sera from infected humans and mice and rabbits immunized with L_3G antigens. Moreover, it is difficult to unexplain why this component (39 KD) gave a rather strong reaction with the angiostrongyliasis serum. It should be mentioned here that this is not a non-specific reaction with any serum protein because it failed to react with the sera from normal humans, mice and rabbits. The results obtained with somatic antigen that was probed with rabbit anti-surface extract showed a strong reactivity at the 44 KD position (Figure 35), indicating that this 44 KD component was a common antigen found in both somatic and surface extract. It should be recalled, however, that this component is not a major somatic protein as determined from its Coomassie blue intensity. In addition, data obtained from the immunoblotting of somatic extract with the sera from infected humans and mice and

rabbits immunized with somatic extract components, showed that this 44 KD component was the most reactive of somatic counterpart. Unfortunately, this immunogenic component also reacted strongly with the serum from angiostrongyliasis patients (Figure 32 B), thus it is of little value in differenial diagnosis for this 2 nematode infections. This immunogenic component (44 KD) was also reported previously by Priwan (16) and Nopparatana *et al.*, (27). Moreover, Priwan (16) reported that this component reacted strongly with the sera from mice infected with other nematodes i.e., *Aspicularis Tetraptera, Syphacia obvelata* and blood flukes i.e., *S. haematobium, S. mansoni and S. japonicum.*

When one compares the immunoblots of somatic extract probed with serum from gnathostomiasis patients with those from angiostrongyliasis patients, one gets the impression that the low molecular weight components at the 26, 21 and 19 KD positions (Figure 32 B) were more or less *Gnathostoma*-specific antigens. Angiostrongyliasis sera reacted more strongly and consistently with the high molecular weight components, particularly those with molecular weight greater than 38 KD. Accordingly, it is easy to differentiate the overall immunoblotting patterns of somatic extract probed with either gnathostomiasis serum or angiostrongyliasis serum.

The immunoblotting patterns of the somatic extract reported in this study are considerably different from those published earlier by Nopparatana *et al.*, (27). In their study, a few low molecular weight components were found to react weakly with the gnathostomiasis sera. In the present study, several of these low molecular weight components reacted strongly with sera from gnatho-

stomiasis patients and the ones with molecular weight of 26, 21 and 19 KD appeared to be *Gnathostoma*-specific antigens. The discrepancy between these 2 studies could be attributable to the different procedures and time employed in electroblotting of proteins onto a nitrocellulose membrane. In this present study, all proteins in the gel were satisfactorily transferred onto a nitrocellulose membrane as shown by comparing the protein pattern on a gel stained with Coomassie blue and Amido black staining of protein on a nitrocellulose after blotting procedure (Figure 32 A and B). We found that the blotting time employed for such a transfer of low moleculor weight components was rather critical as these low molecular weight components could be easily lost if the current was applied too long.

Like their protein staining patterns, the immunoblotting patterns of the ES antigens probed with the serum from infected human and rabbit immunized with ES antigen appeared to be less complex than their somatic counterpart (Figures 34 and 35). In the ES immmunoblots, major components that reacted with these sera were those with the molecular weight range of 65 to 33 KD which were absent in the blots of somatic extract reacted with sera from infected humans and animals except the band with the molecular weight of 44 KD. The 44 KD component in ES preparation reacted strongly with sera from patients infected with gnathostomiasis. However, a similar size component (44 KD) was also found in the somatic counterpart and it also reacted strongly with gnathostomiasis serum. This highly immunogenic component in both antigen preparations had a different biochemical property, in fact, the one present in ES preparation was a glycoprotein, whereas that of

somatic extract was a protein in nature (Figure 31). The exact nature of these 2 components remains to be investigated.

It was surprising to note that the serum specimens from patients with angiostrongyliasis failed to react with any ES component blotted onto a nitrocellulose. This observation suggests that the ES antigen may be a better choice for distinguishing gnathostomiasis from angiostrongyliasis. In fact, additional data obtained from R. Chawengkirttikul showed that a large number of sera from patients with angiostrongyliasis failed to react with L_3G ES antigen in the ELISA. Thus, the ES antigen obtained from an *in vitro* maintenance could be used for differential diagnosis of these 2 parasitic infections which have many other clinical features in common. However, the possibility of a cross-reaction of ES antigen with sera from other parasitic infections has not yet been investigated.

It was previously demonstrated that patients with *Gnathostoma* infection had elevated specific serum IgG and IgE (18, 19, 22) and such a quantitation could be used with a high degree of reliability to confirm the presumptive diagnosis of human gnathostomiasis. However, the specificity of the test has never been critically investigated, particularly in the case of *A. cantonensis* which in many cases causes a disease with similar cerebral signs and symptoms. Therefore, the availability of a more refined antigen is required if one is to have a more satisfactory test. In the present study, data obtained from immunoblotting analysis clearly demonstrated that the L_3G ES antigen was superior to the crude somatic extract in this aspect and it could readily distinguish gnathostomiasis from angiostrongyliasis. In fact, immunoprecipitation

results support the above conclusion (R. Chawengkirttikul, unpublished observations).

In this study, we analyzed both serum and CSF specimens obtained from patients with various forms of cerebral gnathostomiasis including SAH and EM for the presence of antibody reactive with *G. spinigerum* somatic antigen with a main objective of looking for the possibility of using CSF for immunodiagnosis. Furthermore, by comparing the specific antibodies obtained in both serum and CSF of individual patients, particularly when analyzed in conjunction with other immunological parameters, it would be possible to obtain information regarding local antibody synthesis originating from immune components within the CNS. The results presented in Figures 26-27 and Tables 15-17 clearly demonstrated that a large majority of the patients with CNS involvements gave high serum antibodies and moreover antibody could be readily detected in the CSF specimens although most CSF titers were considerably lower than those of the serum. While the specific antibody could not be detected in CSF of one patients with low serum antibody titer, (case no. 32, Table 20) it was noted that there was no patient giving a positive specific antibody in CSF, but giving a negative result in serum. All of these results suggested that CSF specimens can be used for antibody detection in cerebral gnathostomiasis patients if for any reason serum specimens are not available. However, serum is still a better choice as there is a less chance of obtaining a false negative finding.

An attempt was made to analyze data in order to obtain evidence on whether or not *G. spinigerum* could stimulate cells

within the CNS to produce antibodies. A local antibody production within CNS had been proven to occur in multiple sclerosis, inflammatory state, i.e., neurosyphilis, encephalitis, meningitis (51) and other meningeal disorders with parasitic infestation, for instance, angiostrongyliasis (52). In this study, several proteins and a number of immunological parameters, e.g., albumin ratio, IgG-albumin index and specific activity of antibody were used for such an analysis. It was found that five such patients (case no. 2, 7, 10, 11 and 30, Tables 15-20) with CNS involvements exhibited evidence for local antibody synthesis against *G. spinigerum*. However,in only two cases could be confirmed, i.e. by normal albumin ratio, IgG-albumin index above 1.0 and considerably high specific antibody activity in CSF (case no. 2 and 7, Tables 15 and 16). Results obtained from the immunoblotting experiment comparing serum and CSF antibodies reacted with somatic extract are also consistent with the above conclusion. The CSF but not the serum from one of these patients (case no. 2 Table 15) was found to have antibody reactive with a component having molecular weight of 58 KD (data not shown in this study).

The detection of antibodies in either serum or CSF specimens is not the best indication for an active infection with this parasite, because the antibody can persist for a long period of time after the worm is removed from the host. In fact, data obtained from R. Chawengkirttikul showed that specific antibody could persist for approximately six months after the worm had been removed from the host. If one wants to use an immunological technique to monitor the success of anthelminthic treatment or to monitor the activity of the worm one needs to distinguish past from present infection. The

presence of parasite antigen in either the circulation or body fluid would be a better indication for an active infection. Therefore, in this study, attempt was undertaken to develop a sensitive method that could be used to detect any free antigen in the CSF of these patients. The reason that CSF was used is that this nematode infection often involves CNS and therefore the CSF is the most obvious choice. If the parasite is present within the CNS and is metabolically active, it would be expected that the ES antigen should be present in large quantities in the CSF, particularly when one considers the dilution factor by the CSF compared with that of the serum. The total volume of CSF in normal adults is about 140-150 ml (48), while that of the blood is about 4-5 liters. Therefore, if one considers a dilution factor alone, CSF is by for a better choice to use as a specimen for detection of antigen for this parasitic infection.

In the previous section, it was indicated that one larva may produce about 700 ng of ES proteins per day in an *in vitro* condition. If it is assumed that this is an optimum ES production, then one worm should produce about 5 ng per millilitre of CSF per day. However, such an amount was estimated while the worm was maintained in an *in vitro* condition and this concentration is probably underestimated considering the worm in its natural environment. Therefore, the estimation of the metabolic product secreted by one larva in the *in vitro* condition (5 ng/ml/day) is probably a minimal estimation. In this present study, a highly sensitive B-SA ELISA technique for a detection of as little as 2 ng/ml of ES protein (Figure 20, Table 5) was developed. In addition, the specificity of

this sensitive method was also tested with various parasite antigens, including *O. viverrini, S. mansoni, T. crassiceps, T. hydatigena* and *A. cantonensis* third stage larvae and adult, and it was found that this ELISA method was quite specific for the detection of *Gnathostoma* ES antigen (Figure 23). When one considers the sensitivity of B-SA ELISA used, it should be possible to detect the presence of free antigen in CSF of cerebral gnathostomiasis patients if it is present in free state. In fact, only one case out of twenty-eight patients (case no. 6, Table 8) gave a definitively positive result. One possible reason for inability to detect the presence of any free antigen in most patients is the presence of antibodies in the CSF. These antibodies may react with any free antigen that might be present in these CSF specimens, thus resulting the formation of antigen-antibody complexes in the CSF of these patients. In fact, the immune complexes were detected in one CSF specimen (case no. 10, Table 23). It should be noted that no antibodies could be detected in the CSF specimen of the one patient (case no. 6, Table 16) who was found to have antigen in his CSF. This evidence, therefore, supports the explanation given above. However, it is possible to detect antigen in the immune complexes if the latter is dissociated by a suitable method prior to being quantitated. Such a dissociation method has been reported by Bowman *et al.*, (59) who detected the circulatory excretory-secretory antigen levels of *T. canis* in the sera from experimentally infected mice. The individual mouse serum was heat-EDTA treated to dissociate the antigen-antibody complexes prior to being used for antigen detection. Results showed that all of the treated sera had significantly

higher ES antigen concentration than the untreated ones. Such an approach may be applied for our study. However, the lability of *Gnathostoma* antigens has to be analyzed prior to being used.

It is possible to avoid the above problem by developing a method to detect the parasite component(s) that cannot stimulate antibody production in humans. Such a method could make used of antibody against this component produced artificially in some suitable experimental animals. For instance, one may experimentally prepare antibodies against a purified or partially purified secretory component which is not immunogenic in natural infection in humans but could be made immunogenic following immunization with strong adjuvant, i.e., Complete Freund's adjuvant.

The other possible explanation for a negative finding may be the use of inappropriate L_3G stage. In most cases of cerebral gnathostomiasis, either an immature or an adult male worm was obtained by surgery or at autopsy from the brains of patients died of cerebral gnathostomiasis (8, 9, 60), while in the present study, the advanced third-stage larvae were used as an antigen for the preparation of specific antibody to capture the L_3G antigen in CSF. In some earlier work with other parasites, although it was demonstrated that there was a restricted number of stage and species specific antigens in the secretions of *T. spiralis* (57). There were also antigens common to all stages. However, because the immunoblotting patterns of ES antigen probed with sera from patients with gnathostomiasis exhibited strong reactions, such a possibility is unlikely for this infection.

In conclusion, the detection of antigen in CSF by B-SA ELISA is not suitable for immunodiagnosis of cerebral gnathostomiasis. Alternatively, the quantitation of specific IgG antibodies in serum, particularly using the more refined ES component as a diagnostic antigen, appears to be the most reliable laboratory test to diagnose or to confirm a presumptive diagnosis of cerebral gnathostomiasis. Either serum or CSF specimens can be used for antibody detection, although the use of serum specimens appears to be more reliability than the use of CSF specimens.

CHAPTER VI

Summary

The various *G. spinigerum* antigen preparations including somatic, ES and surface extract were characterized, employing a number of physicochemical and immunological methods in order to define specific component(s) with potential for immunodiagnosis of gnathostomiasis. The SDS-PAGE pattern of the somatic antigen was highly complex consisting of proteins and glycoprotein with molecular weight ranging from more than 116 to 13 KD. On the other hand, the ES antigen and surface extract consisted of components with more restricted molecular weight ranges of 98 to 12 KD and 70 to 16 KD respectively. The predominant somatic component with a molecular weight of 38 KD was the only major glycoprotein detected in the somatic extract, as demonstrated by concanavalin-A. On the contrary, a majority of the ES products particularly those with molecular weight range of 55 to 40 KD, were glycoproteins. The immunoenzymatic blotting patterns of both somatic and ES antigens showed strong reaction with sera from infected humans and mice and from rabbits immunized with various L_3G antigen preparations. The immunoenzymatic blotting pattern of somatic antigen reacting with serum from rabbit immunized with surface extract suggested that the 44 KD component in the somatic antigen represented a surface component of the parasite. Specificity of these two antigens was analyzed with angiostrongyliasis serum obtained from patients suspected of having been infected with *A. cantonensis*, a common nematode found within CNS. The immunoblotting pattern showed that the low molecular weight components of somatic antigen (26, 21 and

19 KD) reacted specifically with gnathostomiasis sera. On the other hand, those of high molecular weight components (more than 38 KD), particularly the major predominant component (39 KD) reacted strongly with the angiostrongyliasis serum. On the contrary, the ES antigens blotted onto a nitrocellulose membrane failed to react with angiostrongyliasis serum. Thus, the ES antigen appears to be *Gnathostoma*-specific component and may have potential for differential immunodiagnosis of gnathostomiasis and angiostrongyliasis.

An attempt was also undertaken to detect ES antigen in cerebrospinal fluid (CSF) of the patients with cerebral gnathostomiasis in order to monitor the activity of the worm. The biotin-streptavidin (B-SA) ELISA used for such a detection could detect the presence of antigen to a level of 2 ng protein/ml and was found to be specific for *Gnathostoma* ES antigen. With this test, only one of the twenty-eight patients showed a positive (free) antigen in his CSF specimen. It should be indicated that no antibody could be detected in this CSF specimen. With other patients, a high level of antibody was present in the CSF, therefore, any antigen that may be produced and secreted must be in a form of immune complexes. In fact, in one of these patients the immune complexes were detected in his CSF.

In conjunction with the other protein indices including albumin ratio, IgG-albumin index and specific antibody activity, this data suggest that in addition to serum antibody which may reach CSF in various degrees, local production of anti-*G. spinigerum* antibody does occur within the CNS of some patients with cerebral gnathostomiasis.

In conclusion, using B-SA ELISA for the detection of *Gnathostoma* antigen is not suitable for the immunodiagnosis of gnathostomiasis. Alternatively, the detection of specific antibody is probably more effective in term of serodiagnosis, particularly when the ES component is available. The latter failed to react with angiostrongyliasis serum in immunoblotting and therefore using it as antigen would provide one with a reliable technique for differential diagnosis of gnathostomiasis and angiostrongyliasis.

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APPENDIX

1% Acid-pepsin

Pepsin A (BDH, England)	10.0	gm
Concentrated (11.6 N) HCl	7.0	ml
Distilled water to	1.0	liter

Hank's balanced salt solution (HBSS)

Stock A:	NaCl	168.0	gm
	KCl	8.0	gm
	$MgSO_4.7H_2O$	2.0	gm
	$MgCl_2.6H_2O$	2.0	gm
	$CaCl_2$	2.0	gm
	Tridistilled water to	1.0	liter
	Sterilization by 0.45 µm millipore membrane filtration		
Stock B:	Na_2HPO_4	1.2	gm
	KH_2PO_4	1.2	gm
	Dextrose (Bacto)	20.0	gm
	Chloroform	2.0	ml
	Tridistilled water to	1.0	liter
	Sterilization by 0.45 µm millipore membrane filtration		
Working HBSS:	Stock A	1	part
	Stock B	1	part
	Sterile distilled water	18	part

BME medium 1X

BME powder 1 package (Eagle's salts)	9.2	gm
Tridistilled water to	1.0	liter
Adjusting pH with $NaHCO_3$ to 7.2-7.4		
Sterilization by 0.45 µm millipore membrane filtration		

Sample buffer 5x pH 6.8

Trizma base (Sigma)	0.3784	gm
SDS	0.50	gm
Glycerol	5.0	ml
2-mercaptoethanol	2.5	ml
Bromphenol blue	0.005	gm
Distilled water to	10.0	ml

Stock acrylamide (30%)

Acrylamide (Sigma)	30.0	gm
N,N-bis-methylene acrylamide (Sigma)	0.8	gm
Distilled water to	100.0	ml

Gel buffer pH 8.9 (for small gel)

Trizma base (Sigma) (0.375 M)	9.075	gm
1N HCl	12.0	ml
Distilled water to	25.0	ml

Gel buffer pH 6.8 (for small gel)

Trizma base (Sigma) (0.125 M)	3.0275	gm
Adjusted pH with concentrated HCl		
Distilled water to	50.0	ml

Electrophoresis buffer pH 8.3

Trizma base (Sigma)	3.0	gm
Glycine	14.4	gm
SDS	1.0	gm
Distilled water to	1.0	liter

Coomassie blue stain

Coomassie brilliant blue R	0.2	gm
Glacial acetic acid	7.0	ml
Methanol	46.5	ml
Distilled water	46.5	ml

Destaining solution

Methanol	50.0	ml
Glacial acetic acid	70.0	ml
Distilled water to	1.0	liter

50% Methanol and 12% acetic acid

Absolute methanol	500.0	ml
Glacial acetic acid	120.0	ml
Distilled water to	1.0	liter

10% ethanol and 5% acetic acid

95% ethanol	105.0	ml
Glacial acetic acid	50.0	ml
Distilled water to	1.0	liter

0.0034 M potassium dichromate and 0.0032 N nitric acid

Potassium dichromate	1.0	gm
Nitric acid	63.0	ml
Distilled water to	1.0	liter

0.012 M silver nitrate

Silver nitrate (Lab center, Bangkok)	0.41	gm
Deionized water to	200.0	ml

Image developer: 0.28 M Sodium carbonate

Sodium carbonate	29.7	gm
Commercial formalin	0.5	ml
Distilled water to	1.0	liter

0.15 M phosphate buffered saline pH 7.2

NaCl	8.0	gm
KCl	0.2	gm
Na_2HPO_4	1.15	gm
KH_2PO_4	0.2	gm
Distilled water to	1.0	liter

Towbin's buffer

Trizma base (Sigma)	3.035	gm
Glycine	14.413	gm
Methanol	200.0	ml
Distilled water to	1.0	liter

Phosphate buffered saline pH 7.1 with 1% TWEEN 20 for immunoblotting

NaCl	8.5	gm
Na_2HPO_4	1.07	gm
$NaH_2PO_4.2H_2O$	0.39	gm
Distilled water to	1.0	liter
Tween 20	10.0	ml

Substrate buffer for immunoblotting (Alkaline phosphatase)

0.2 M $NaCO_3$	9.0	ml
0.2 M $NaHCO_3$	16.0	ml
0.5 M $MgCl_2$	0.1	ml
Distilled water to	100.0	ml

Substrate solution for immunoblotting (Alkaline phosphatase)

1. O-dianisidine tetrazotized (Sigma)	60.0	mg
Substrate buffer	10.0	ml
2. β-naphthyl phosphate (Sigma)	10.0	mg
Substrate buffer	10.0	ml

mix 1 and 2 solution together and filtered before used

Amido black staining

Amido Schawartz (Sigma)	1.0	gm
Absolute methanol	450.0	ml
Glacial acetic acid	100.0	ml
Distilled water to	1.0	liter

Destaining solution for Amido black staining

Absolute methanol	450.0	ml
Glacial acetic acid	100.0	ml
Distilled water to	1.0	liter

Substrate buffer pH 7.4 for glycoprotein stainling (Horseradish-peroxidase)

Trizma base	6.06	gm
Adjusting pH with 1 N HCl		
Distilled water to	1.0	liter

Substrate working solution for glycoprotein staining

Diaminobenzidine (Sigma)	6.0	mg
Substrate buffer	20.0	ml
35% H_2O_2	30.0	µl

Coating buffer for ELISA 0.05 M Carbonate buffer, pH 9.6

Na_2CO_3	1.59	gm
$NaHCO_3$	2.93	gm
Distilled water to	1.0	liter

Incubation buffer for ELISA

NaCl	89.0	gm
Na_2HPO_4	1.28	gm
NaH_2PO_4 $2H_2O$	0.15	gm
Tween 20	0.5	ml
Distilled water to	1.0	liter

Washing buffer for ELISA

NaCl	45.0	gm
Tween 20	2.5	ml
Distilled water to	5.0	liter

Substrate buffer Phosphate-citrate buffer, pH 5.0

Na_2HPO_4	7.19	gm
Citric acid	5.19	gm
Distilled water to	1.0	liter

Substrate stock

Orthophenylene diamine (Sigma)	100.0	mg
Methanol	10.0	ml

Substrate working solution

Substrate buffer	19.8	ml
Substrate stock	200.0	µl
3% H_2O_2	20.0	µl

Phosphate buffer pH 8.0 (0.02 M)

1. K_2HPO_4	3.48	gm
Distilled water to	1.0	liter
2. KH_2PO_4	2.72	gm
Distilled water to	1.0	liter
Adjusted 1 with 2 to obtain pH 8.0		

Preparation of DEAE cellulose

Pre-swollen DEAE cellulose was suspended in distilled water and fine particles were removed by slowly decanting the supernatant fluid after larger particles had settled. Then, the cellulose was equilibrated with 0.02 M phosphate buffer pH 8.0 and adjusted to pH 8.0 by phosphoric acid then allow it to equilibate at 4°C overnight. The cellulose was subsequently washed several times with 0.02 M phosphate buffer before packing.

To generate used cellulose, the used cellulose was stirred in 15 volumes of 0.5 N HCl for 30 min and washed in a sinterred glass funnel with distilled water until the pH of the washing solution approaching 4.0. The cellulose was then resuspended in 15 volume of 0.5 N NaOH of for 30 min and washed again with distilled water until the pH was neutral. It was equilibrated in 0.02 M phosphate buffer pH 8.0 before packing.

Veronal buffer stock solution 5x (Complement fixing diluent) pH 7.2-7.4

NaCl	41.90	gm
$NaHCO_3$	1.26	gm
Na barbital	1.50	gm
Barbital	2.30	gm
$MgCl. 6H_2O$	0.50	gm
$CaCl_2. 2H_2O$	0.10	gm
Distilled water to	1.0	liter

Gelatin veronal buffer (GVB)

Gelatin (Merck)	0.1	gm
Stock veronal buffer	20.0	ml
Distilled water to	100.0	ml

Alsever's solution pH 6.1

Dextrose	2.05	gm
Sodium citrate	0.80	gm
Sodium chloride	0.42	gm
Distilled water to	100.0	ml

Sterilization by 0.45 µm millipore membrane filtration

Sheep red cells

Sheep red cells	10.0	ml
Alsever's solution	10.0	ml

Sensitized sheep red blood cells

5% sheep red cells in working veronal buffer	5.0	ml
Anti-sheep hemolysin (BBL, Maryland, U.S.A)	25.0	µl
Working veronal buffer to	10.0	ml

Borate buffered saline pH 8.4

Boric acid	6.18	gm
Disodium tetraborate	9.54	gm
Sodium chloride	4.38	gm
Distilled water to	1.0	liter

12.5% PEG 6000

PEG 6000	12.5	gm
Borate buffered saline	100.0	ml

Statistical Analysis

Kruskal-Wallis test used for comparision the mean value of at least three independent samples and when each group of sample has small observations.

All observations in three (or more) independent samples are arranged in an array of ascending order and then ranked together to the combined array.

where k = number of independent samples

n_i = sample size in the sample (i)

N = Σn_i

R_i = the sum of the rank number of each sample (i)

t_i = the number of the ties ranks

ΣT_i = $\Sigma(t_i^3 - t_i)$

1. If there are not any tied rank: the formula for calculation is

$$H = \frac{12\Sigma \frac{R_i}{n_i}}{N(N+1)} - 3(N+1)$$

2. If there are some substantial number of ties, the adjusted test is

$$H = \frac{\frac{12}{N(N+1)} \quad \Sigma\frac{R_i}{n_i} - 3(N+1)}{1 - \frac{\Sigma T_i}{N^3-N}}$$

Multiple Comparisons

When a hypothesis-testing procedure such as the Kruskal-Wallis test leads to reject the null hypothesis and thus to conclude that not all sampled populations are identical, the multiple comparison test is used to test which populations are different from which others.

where

$\bar{R}_i$ = mean of the ranks of i^{th} sample

$\bar{R}_j$ = mean of the ranks of j^{th} sample

z = $\alpha/k\,(k-1)$; α is level of significance

1. If there are not any tied rank: the inequality formula is

$$\left|\bar{R}_i - \bar{R}_j\right| \leqslant Z(1-[\alpha/k(k-1)])\sqrt{\frac{N(N+1)}{12}\left(\frac{1}{n_i} + \frac{1}{n_j}\right)}$$

2. If ther are extensive ties in the data, the adjusted inequality is

$$\left|\bar{R}_i - \bar{R}_j\right| \leqslant Z_{(1-[\alpha/k(k-1)]}\sqrt{\frac{[N(N^2-1) - (\Sigma t^3-\Sigma t)]\left[\frac{1}{n_i} + \frac{1}{n_j}\right]}{12(N-1)}}$$

Any difference $\left|\bar{R}_i - \bar{R}_j\right|$ that is larger than the right-hand side of inequality formular is declared significant at the α level.

1. Specific IgG antibody titers to somatic antigen in serum (log_{10}) of 4 groups of patients (CNS-G, EM, UM and other CNS involvements groups).

Group I (CNS-G)	4.91, 4.93, 5.62, 4.66, 5.81
Group II (EM)	4.70, 3.38, 4.69, 3.92
Group III (UM)	2.92, 2.10, 2.80
Group IV (other CNS involvements)	2.80, 2.58, 3.10, 2.10, 2.90, 2.67, 2.38
	2.23, 2.72, 2.65, 2.69, 2.79, 2.69, 2.74

Hypotheses H_0 : The four populations represent identical data

H_1 : The four populations do not represent identical data

at significant level (α) = 0.05

1.1 Ranks the data together

Group I	23, 24, 25, 20, 26
Group II	22, 18, 21, 19
Group III	16, 1, 13.5
Group IV	13.5, 5, 17, 2, 15, 7, 4
	3, 10, 6, 8.5, 12, 8.5, 11

$n_1 = 5$	$R_1 = 118$	$R_1 = 23.6$
$n_2 = 4$	$R_2 = 80$	$R_2 = 20$
$n_3 = 3$	$R_3 = 30.5$	$R_3 = 10.17$
$n_4 = 14$	$R_4 = 122.5$	$R_4 = 8.75$

$N = n_1 + n_2 + n_3 + n_4 = 26$

1.2 Considering the tied rank

at titers (i) 2.69, 2.8

number of tie 2, 2

$$\Sigma T_i = \Sigma(t_i^3 - t_i) = 2(2^3 - 2) = 12$$

$$H = \frac{\frac{12}{N(N+1)} \frac{R_i^2}{n_i} - 3(N+1)}{1 - \frac{\Sigma T_i}{N^3-N}}$$

$$= \frac{\frac{12}{26(26+1)} \left[\frac{118^2}{5} + \frac{80^2}{4} + \frac{30.5^2}{3} + \frac{122.5^2}{14}\right] - 3(26+1)}{1 - \frac{12}{26^3-26}}$$

$$= 17.576$$

$H \simeq \chi^2$, degree of freedom (ν) = k-1 = 3

In Table χ^2: χ^2 = 12.84 P-value = 0.005

$\therefore$ $P < \alpha$ rejected H_0 at $\alpha = 0.05$

Conclusion : The four populations do not represent identical data at significant level 0.05 ($P < 0.05$).

Multiple Comparison

$\bar{R}_1 = 23.6$ $\bar{R}_2 = 20.0$ $\bar{R}_3 = 10.17$ $\bar{R}_4 = 8.75$

$n_1 = 5$ $n_2 = 4$ $n_3 = 3$ $n_4 = 14$

If let significant level (α) = 0.05, $k = 4$

$\alpha/k\,(k-1) = 0.05/4(4-1) = 0.0041$

From Table Z: Z value with 0.0041 of the area to its right to be 2.64

1.3 To Compare groups I and II

$$\left|\bar{R}_1 - \bar{R}_2\right| \leqslant Z\sqrt{\frac{N(N+1)}{12}\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}$$

$$\left|23.6 - 20.0\right| \leqslant 2.64\sqrt{\frac{9(9+1)}{12}\left(\frac{1}{5} + \frac{1}{4}\right)}$$

$$3.60 < 4.850$$

Conclusion: The populations represented by samples I and II are not significantly different at level 0.05.

1.4 To compare groups I and III

$$\left|\bar{R}_1 - \bar{R}_3\right| \leqslant Z\sqrt{\frac{N(N+1)}{12}\frac{1}{n_1} + \frac{1}{n_3}}$$

$$\left|23.60 - 10.17\right| \leqslant 2.64\sqrt{\frac{8(8+1)}{}\frac{1}{5} + \frac{1}{3}}$$

$$13.43 > 4.710$$

Conclusion: The populations represented by samples I and III are significantly difference at level 0.05.

1.5 To compare groups I and IV

at titer 2.69; number of tie = 2

$$\left| \bar{R}_1 - \bar{R}_4 \right| \leq Z \sqrt{\frac{[N(N^2-1) - (\Sigma t^3 - \Sigma t)]\left[\frac{1}{n_1} + \frac{1}{n_4}\right]}{12(N-1)}}$$

$$\left| 23.60 - 8.75 \right| \leq 2.64 \sqrt{\frac{[19(19^2-1) - (2^3-2)]\left[\frac{1}{5} + \frac{1}{14}\right]}{12(19-1)}}$$

$$14.85 > 7.730$$

Conclusion: The populations represented by samples I and IV are significantly different at level 0.05

1.6 To compare groups II and III

$$\left| \bar{R}2 - \bar{R}3 \right| \leq Z \sqrt{\frac{N(N+1)}{12}\left(\frac{1}{n_2} + \frac{1}{n_3}\right)}$$

$$\left| 20.0 - 10.17 \right| \leq 2.64 \sqrt{\frac{7(7+1)}{12}\left(\frac{1}{4} + \frac{1}{3}\right)}$$

$$9.83 > 4.343$$

Conclusion: The populations represented by samples II and III are significantly different at level 0.05.

1.7 To compare groups II and IV

at titer 2.69; number of tie = 2

$$\left| \bar{R}_2 - \bar{R}_4 \right| \leq Z \sqrt{\frac{[N(N^2-1) - (\Sigma t^3 - \Sigma t)]\left[\frac{1}{n_2} + \frac{1}{n_4}\right]}{12(N-1)}}$$

$$\left| 20.0 - 8.75 \right| \leq 2.64 \sqrt{\frac{[18(18^2-1) - (2^3-2)]\left[\frac{1}{4} + \frac{1}{14}\right]}{12(18-1)}}$$

$$11.25 > 7.986$$

Conclusion: The populations represented by samples II and IV are significantly difference at level 0.05.

1.8 To compare groups III and IV

at titer 2.8 2.69

number of tie 2 2

$$\left|\bar{R}_3 - \bar{R}_4\right| \leq Z\sqrt{\frac{[N(N^2-1) - (\Sigma t^3 - \Sigma t)]\left[\frac{1}{n_3} + \frac{1}{n_4}\right]}{12(N-1)}}$$

$$\left|10.17 - 8.75\right| \leq 2.64\sqrt{\frac{[17(17^2-1) - 2(2^3-2)]\left[\frac{1}{3} + \frac{1}{4}\right]}{12(17-1)}}$$

$$1.42 < 8.467$$

Conclusion: The populations represented by samples III and IV are not significantly different at level 0.05.

2. Specific IgG antibody titers to somatic antigen in CSF ($\log_{10}$) of 4 groups of patients (CNS-G, EM, UM and other CNS involvements groups)

Group I (CNS-G)	3.30, 3.67, 3.40, 1.92, 4.78
Group II (EM)	2.76, 1.34, 3.78, 2.81,
Group III (UM)	0.69, <0.69, <0.69
Group IV (other CNS involvements)	<0.69, <0.69, <0.69, <0.69, <0.69, <0.69, <0.69
	<0.69, 0.78, <0.69, <0.69, <0.69, <0.69, <0.69

Hypotheses H_0 : The four populations represent identical data.

H_1 : The four populations do not represent identical data at significant level (α) = 0.05

2.1 Ranks the data together

Group I	22,	24,	23,	19,	26		
Group II	20,	18,	25,	21			
Group III	16,	8,	8				
Group IV	8,	8,	8,	8,	8,	8,	8
	8,	17,	8,	8,	8,	8,	8

$n_1 = 5$ $R_1 = 114$ $\bar{R}_1 = 22.8$

$n_2 = 4$ $R_2 = 85$ $\bar{R}_2 = 21.25$

$n_3 = 3$ $R_3 = 32$ $\bar{R}_3 = 10.67$

$n_4 = 14$ $R_4 = 121$ $\bar{R}_4 = 8.64$

$N = n_1 + n_2 + n_3 + n_4 = 26$

2.2 Considering the tied rank

at titer < 0.69

number of tie 15

$$\Sigma T_i = \Sigma(15^3 - 15) = 3,360$$

$$H = \frac{\frac{12}{N(N+1)} \Sigma \frac{R_1^2}{n_1} - 3(N+1)}{1 - \frac{\Sigma T_i}{N^3-N}}$$

$$= \frac{\frac{12}{26(26+1)}\left[\frac{114^2}{5} + \frac{85^2}{4} + \frac{32^2}{3} + \frac{121^2}{14}\right] - 3(26+1)}{1 - \frac{3,360}{26^3-26}}$$

$$= 22.27$$

$H \approx \chi^2$ at degree of freedom (ν) $= k-1 = 3$

In Table χ^2: $\chi^2 = 12.84$ $P = 0.005$

$\therefore P < \alpha$ rejected H_0 at $\alpha = 0.05$

Conclusion: The four populations do not represent identical data at significant level 0.05 ($P < 0.05$)

Multiple Comparison

$\bar{R}_1 = 22.8$ $\bar{R}_2 = 21.25$ $\bar{R}_3 = 10.67$ $\bar{R}_4 = 8.64$

$n_1 = 5$ $n_2 = 4$ $n_3 = 3$ $n_4 = 14$

If let significant level (α) = 0.05, $k = 4$

$\alpha/k(k-1) = 0.05/4(4-1) = 0.0041$

From Table Z: Z value with 0.0041 of the area to its right to be 2.64

2.3 To compare group I and II

$$\left|\bar{R}_1 - \bar{R}_2\right| \leqslant Z\sqrt{\frac{N(N+1)}{12}\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}$$

$$\left|22.8 - 21.25\right| \leqslant 2.64\sqrt{\frac{9(9+1)}{12}\left(\frac{1}{5} + \frac{1}{4}\right)}$$

$$1.55 < 4.85$$

Conclusion: The populations represented by samples I and II are not significantly different at level 0.05

2.4 To compare groups I and III

at titer < 0.69

number of tie 2

$$\left|\bar{R}_1 - \bar{R}_3\right| \leqslant Z\sqrt{\frac{[N(N^2-1) - (\Sigma t^3 - \Sigma t)]\left[\frac{1}{n_1} + \frac{1}{n_3}\right]}{12(N-1)}}$$

$$\left|22.80 - 10.67\right| \leqslant 2.64\sqrt{\frac{[8(8^2-1) - (2^3-2)]\left[\frac{1}{5} + \frac{1}{3}\right]}{12(8-1)}}$$

$$12.13 > 4.69$$

Conclusion: The populations represented by samples I and III are significantly different at level 0.05.

2.5 To compare groups I and IV

at titer $<$ 0.69

number of tie 13

$$\left|\bar{R}_1 - \bar{R}_4\right| \leqslant Z\sqrt{\frac{[N(N^2-1) - (\Sigma t^3-\Sigma t)]\left[\frac{1}{n_1} + \frac{1}{n_4}\right]}{12(N-1)}}$$

$$\left|22.8-8.64\right| \leqslant 2.64\sqrt{\frac{[19(19^2-1) - (13^2-13)]\left[\frac{1}{5} + \frac{1}{14}\right]}{12(19-1)}}$$

$$14.16 > 6.38$$

Conclusion: The populations represented by samples I and IV are significantly different at level 0.05.

2.6 To compare groups II and III

at titer $<$ 0.69

number of tie 2

$$\left|\bar{R}_2 - \bar{R}_3\right| \leqslant Z\sqrt{\frac{[N(N^2-1) - (\Sigma t^3-\Sigma t)\left[\frac{1}{n_2} + \frac{1}{n_3}\right]}{12(N-1)}}$$

$$\left|21.25-10.67\right| \leqslant 2.64\sqrt{\frac{[(7(7^2-1) - (2^3-2)]\left[\frac{1}{4} + \frac{1}{3}\right]}{12(7-1)}}$$

$$10.58 > 4.315$$

Conclusion: The populations represented by samples II and III are significantly different at level 0.05.

2.7 To compare groups II and IV

at titer < 0.69

number of tie 13

$$\bar{R}_2 - \bar{R}_4 \leqslant Z\sqrt{\frac{[N(N^2-1) - (\Sigma t^3-\Sigma t)]\left[\frac{1}{n_2} + \frac{1}{n_4}\right]}{12(N-1)}}$$

$$21.25-8.64 \leqslant 2.64\sqrt{\frac{[18(8^2-1)-(13^3-13)]\left[\frac{1}{4} + \frac{1}{14}\right]}{12(18-1)}}$$

$$12.61 > 6.309$$

Conclusion: The populations represented by samples II and IV are significantly different at level 0.05.

2.8 To compare groups III and IV

at titer < 0.69

number of tie 15

$$\left| \bar{R}_3 - \bar{R}_4 \right| \leqslant Z\sqrt{\frac{[N(N^2-1) - (\Sigma t^3 - \Sigma t)\left[\frac{1}{n_3} + \frac{1}{n_4}\right]}{12(N-1)}}$$

$$\left|10.67-8.64\right| \leqslant 2.64\sqrt{\frac{[17(17^2-1) - (15^3-15)]\left[\frac{1}{3} + \frac{1}{14}\right]}{12(17-1)}}$$

$$2.03 < 4.746$$

Conclusion: The populations represented by samples III and IV are not significantly different at level 0.05.