GNATHOSTOMA AND GNATHOSTOMIASIS IN THREE PROVINCES OF LAO PDR

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Thesis
entitled

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ABSTRACT

While human gnathostomiasis cases have been reported in Lao PDR since 1975, little is known about this disease in this country. We aimed to investigate human gnathostomiasis and gnathostome species in Lao PDR. One village each in Bokeo in the north, Vientiane in the central, and Champasack in the south of Lao PDR were selected as study sites. Using immunoblot technique, 420 randomely selected and 172 volunteer participants' sera were examined. Overall, 125 (29.8%) out of 420 sera of the randomly selected participants and 25 (14.5%) of 172 sera of volunteers were found to be seropositive having anti-*Gnathostoma* IgG antibody against 24 kDa fraction. Sero-prevalence of each province were quite variable with high positive rates of 47.1% in Naxon, Vientiane and 38.6% in Nongtearnoy, Champasack Provinces and a low positive rate of 3.6% in Phibounthong, Bokeo Province. When *Gnathostoma* infection-related risk factors were assessed using a questionnaire with closed questions by the interview for the 420 randomly selected participants, raw/undercooked fish consumption was closely related to seropositive cases in Naxon and Nongtearnoy. Several fish, swamp eels and frogs collected from these two villages were infected with larvae of *Gnathostoma spinigerum*. Gnathostome eggs were found in dogs' feces collected from Nongtearnoy.

KEY WORDS: SEROPREVALENCE / GNATHOSTOMIASIS / *GNATHOSTOMA SPINIGERUM* / FOOD-BORNE NEMATODE / /IMMUNOBLOT / LAO PDR

118 pages

CONTENTS

		Page
ACKNOWLEDGEMENTS		iii
ABSTRACT (ENGLISH)		iv
CONTENTS		v
LIST OF TABLES		viii
LIST OF FIGURES		ix
LIST OF ABBREVIATIONS		xii
CHAPTER I	**INTRODUCTION**	1
CHAPTER II	**OBJECTIVES**	6
2.1	General objective	6
2.2	Specific objective	6
CHAPTER III	**REVIEW OF THE LITERATURES**	7
3.1	General information	7
3.2	History and geographical distribution	10
3.3	Classification of the parasite	12
3.4	Morphology of *Gnathostoma* species	12
3.5	Other *Gnathostoma* species proven to cause human infections	25
3.6	Life cycle	32
3.7	Mode of transmission	38
3.8	Pathology and symptomatology	39
3.9	Diagnosis approaches	43
3.10	Immunodiagnosis of human gnathostomiasis	43
3.11	Treatment	45
3.12	Prevention	45
3.13	Other *Gnathostoma* species	45
3.14	*Gnathostoma* specific confirmation of the AL3	49
3.15	Immune responses in human gnathostomiasis	50

CONTENTS (cont.)

		Page
CHAPTER IV	**MATERIALS AND METHODS**	52
4.1	Study site	52
4.2	Study design and population	54
4.3	Sample size estimation	55
4.4	Data collection	56
4.5	Preparation of *G. spinigerum* antigens	57
4.6	Immunoblot technique	59
4.7	Molecular weight determination	61
4.8	Data analysis	62
4.9	Treatment for the participants	62
4.10	Ethical consideration	63
4.11	Significance of the research	63
4.12	Variables	64
4.13	Conceptual framework	65
CHAPTER V	**RESULTS**	66
5.1	Preliminary survey for gnathostomiasis cases	66
5.2	Study population	67
5.3	Prevalence of *Gnathostoma* infection	69
5.4	Risk factors analyze for *Gnathostoma* infection	74
5.5	Intermediate and definitive hosts survey	82
CHAPTER VI	**DISCUSSION**	90
CHAPTER VII	**CONCLUSION AND RECOMMENDATION**	94
7.1	Conclusions	94
7.2	Recommendations	94
REFERENCES		96
APPENDICES		107
Appendix A	Parasite collection	108

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CONTENTS (cont.)

		Page
Appendix B	Protein determination	109
Appendix C	Sodium dodecyl sulphate-polyacrylamine gradient gel electrophoresis (SDS-PAGE)	110
Appendix D	Questionnaire	113
BIOGRAPHY		118

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LIST OF TABLES

Table		Page
1.1	Immunodiagnosed gnathostomiasis involved Lao PDR	4
3.1	The first records of *Gnathostoma* spp. in the literature up to 1991	8
3.2	Currently accepted *Gnathostoma* spp. with reference to human infection	9
3.3	Clinical features of human gnathostomiasis caused by different species	43
5.1	Sera analysis of preliminary survey for gnathostomiasis cases	66
5.2	Characteristics of the randomly selected population in three villages	68
5.3	Characteristics of voluntary participants in three villages	69
5.4	Sero-prevalence of *Gnathostoma* infection in villages	70
5.5	Proportion of raw/undercooked food consumption in villages	75
5.6	Proportion of raw/undercooked foods consumers	76
5.7	Bivariate analysis of some potential risk factors for *Gnathostoma* infection in villages	78
5.8	Multivariable analysis of selected risk factors for *Gnathostoma* infection	80
5.9	Relationship between sero-positivity and the case histories of skin	82
5.10	Prevalence of GsAL3 in intermediate hosts in three villages	83
5.11	Numbers of fecal samples of dog dropped positive for *Gnathostoma spinigerum* eggs in villages	87

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LIST OF FIGURES

Figure		Page
3.1	*G. spinigerum*: females (F) and males (M)	13
3.2	*G. spinigerum* adult, with a cephalic bulb armed with 8 rows of hooks.	13
3.3	Cuticular spines of the posterior body part. SEM of the cuticular armature of *G. spinigerum* from cats in Laos	14
3.4	Details of cuticular spines of the anterior body part. SEM of the cuticular armature of *G. spinigerum* from cats in Laos	14
3.5	Detail of nondendiculated cuticular spines. SEM of the cuticular armature of *G. spinigerum* from cats in Laos	14
3.6	Details of cuticular spines of the anterior body part. SEM of the cuticular armature of *G. spinigerum* from cats in Laos	14
3.7	*G. spinigerum*: Enlarged head-bulb. A pair of lips in the center and four ballonets around them. Generally, eight rows of hooks are aligned	15
3.8	*G. spinigerum*: Anterior view of the swollen head-bulb	15
3.9	*G. spinigerum*: Body surface of the female	16
3.10	*G. spinigerum*: Tail end of a male in ventral view	16
3.11	*G. spinigerum*: Ventral view of the caudal ala of a male spicules omitted	16
3.12	*G. spinigerum*: Early third-stage larvae parasitic in the hemocoel of cyclops	19
3.13	*G. spinigerum*: Early third-stage larva removed from the host	19
3.14	*G. spinigerum*: Advanced third-stage larva encysted in the muscle of a fish	20
3.15	*G. spinigerum*: Head-bulb of advanced third-stage larva	21
3.16	*G. spinigerum*: Structure of advanced third-stage larva	22

LIST OF FIGURES (cont.)

Figure		Page
3.17	*G. spinigerum*: 1: Fertilized egg containing an unsegmented ovum It has a thin bulge (C) at one pole. 2: *G. spinigerum* fertilized egg with 2 cells	24
3.18	*G. spinigerum*: Development of fertilized eggs	24
3.19	Adult of *G. hispidum*: 1: Two worms separately in two pores of the gastric wall of a pig. 2: A male removed from the gastric wall, its headbulb being swellen on the top. 3: Headbulb of the adult. SEM (x100)	25
3.20	*G. hispidum*: Extent and shape of cuticular spines of a female collected from a pig in China	26
3.21	*G. hispidum*: Fertilized egg containing an unsegmented ovum yellowish brown, with a plug at one pole	26
3.22	*G. hispidum*: Advanced 3rd-stage larva. 1: Removed from the muscle of a rat; 2: Headbulb; shape of hooks are similar to those of *G. doloresi*, except that those in the last row are smaller	27
3.23	*G. doloresi*: the whole body is covered with spines	29
3.24	*G. doloresi*: Fertilized egg (D) with bulges (arrow) at both poles, G. spinigerum egg (S) with the same magnification	29
3.25	*G. nipponic*um: no spines on the posterior half of the body	30
3.26	*G. nipponic*um: Anterior end of an advanced third-stage larva removed from the muscle of frog. Right: Enlarged headbulb; number of hooks per row are 24, 26 and 32 posteriorly, noted three rows of hooklet	31
3.27	Fertilized (right) and unfertilized (left) eggs of *G. nipponic*um	31
3.28	Life cycle of *Gnathostoma* species	34
4.1	Map of Lao PDR and the study areas, Bokeo, Vientiane Capital and Champasack Province	53
4.2	Procedure of GsAL3 collection	58

LIST OF FIGURES (cont.)

Figure		Page
4.3	Procedure of GsAL3 crude antigen preparation	57
4.4	Calibration curve was constructed using the LMW protein standards	61
5.1	The immunoblot technique for diagnosis of human gnathostomiasis	71
5.2	Sero-prevalence distribution for infection in three provinces of Lao PDR	72
5.3	Left hand mobile intermittent swelling with tenderness of an acute gnathostomiasis patient	73
5.4	*G. spinigerum* advanced-third stage larvae obtained from 2nd intermediate hosts	84
5.5	The typical cephalic hooklets of GsAL3 obtained from 2nd intermediate hosts	85
5.6	The typical cephalic hooklets of GsAL3 obtained from 2nd intermediate hosts	86
5.7	*Gnathostoma* eggs obtained from dog's feces in Nongtearnoy Village (Normal saline) A.*Gnathostoma* egg with one cell; B. *Gnathostoma* egg developed into two cells	88
5.8	*Gnathostoma* eggs obtained from dog's feces in Nongtearnoy Village (Kato-Katz)	89

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LIST OF ABBREVIATIONS

Abbreviation/symbol	Terms
Ab	antibody
AL3	advanced third-stage larva
Ag	antigen
cm	centimeter
CNS	central nervous system
CSF	cerebrospinal fluid
°C	degree celsuis
DW	distilled water
ELISA	enzyme-linked immunosorbent assay
EL3	early third-stage larva
e.g.	for example
et al.	et alibi and other
etc	etcetera
>	greater
<	smaller
GsAL3	*Gnathostoma spinigerum* advanced third-stage larvae
hr (s)	hour (s)
IB	immunoblot
Ig	immunoglobulin
IgE	immunoglobulin E
IgG	immunoglobulin G
IgM	immunoglobulin M
kDa	kilodalton
kg	kilogram
<	lesser

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LIST OF ABBREVIATIONS (cont.)

Abbreviation/symbol	Terms
LMW	low molecular weight
µm	micrometer
µg	microgram
µg/ml	microgram per milliliter
µl	micro liter
µg	microgram
mA	milliampere
mAb	monoclonal antibody
mg	milligram
mg/ml	milligram per milliliter
min	minute
ml	milliliter
mm	millimeter
MW	molecular weight
%	percent
rpm	revolution per minute
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sec	second
spp.	species
U	unit
U/µl	unit per micro liter

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CHAPTER I
INTRODUCTION

Gnathostoma is the nematode worm parasite in the stomach and other organs of animals. Human gnathostomiasis caused by larval stage of *Gnathostoma* spp., is a food-borne parasitic zoonosis, and was first described in human by Levinsen in 1889 from Thai woman living in Bangkok, Thailand (Daengsvang, 1980; Daengsvang, 1983; Feinstein, 1984). Fifty years earlier (1836), Richard Owen had discovered and described *Gnathostoma spinigerum* in the stomach of a young tiger that died after rupture of its aorta in the London Zoo. The next case of *Gnathostoma* in humans was not reported until 1934 (Ligon, 2005). A variety of names has been used for the disease including larva migrans profundus, nodular migratory eosinophilic panniculitis, Yangtze River edema, consular disease, Shanghai rheumatism, Woodbury bug and Tau-Chit (Daengsvang, 1980; Rusnak et al., 1993). It is a systemic infection caused by migrating nematode larvae of several species of the genus *Gnathostoma* (Nematoda), especially *G. spinigerum* (Daengsvang, 1980; Rusnak et al., 1993; Chai et al., 2003). A total of 13 *Gnathostoma* spp. have been described, human infection reported with five species, *G. spinigerum*, *G. hispidum*, *G. doloresi*, *G. nipponicum*, and *G. binucleatum*. These worms have been reported throughout the world (Daengsvang, 1980, 1982; Miyazaki, 1991; Almeyda-Artigas, 1991; Rusnak et al., 1993; Camacho et al., 1998; McCarthy et al., 2000; Nawa et al., 2004). Species *G. spinigerum* is the most frequent cause of human disease in Asia (Daengsvang, 1980; Rusnak et al., 1993; Rojekittikhun, 2005).

Gnathostomiasis is a well-known parasitosis in several countries in South East Asia (Daengsvang, 1981; Rojekittikhun, 2005) and Latin America (Camacho et al., 2003; Waikagul and Diaz-Camacho, 2007), and increasing reports in European countries (Giudice, 2001; Hennies, 2006) or more recently in Africa (Herman et al., 2009; Hale et al., 2003). Humans acts as an accidental host and acquires the disease

mainly by ingesting raw or insufficiently cooked freshwater fish infected with the encysted advanced third-stage larvae (AL3) and also meat of animal hosts (fish, amphibians, reptiles, birds and mammals). After ingestion, the larvae traverse through the gastric or intestinal wall and migrate into different tissues without further development. (Daengsvang, 1981; Bunnag, 2000; Rojekittikhun, 2005). Human gnathostomiasis is a parasitic zoonosis. From the medical standpoint, *G. spinigerum* is considered to be the most important species in Asia (Daengsvang, 1980; Rusnak et al., 1993; Rojekittikhun, 2005). The clinical manifestations of the disease, also known as larva migrant, are cutaneous (Daengsvang, 1981), ocular (Chen, 1949; Seal, 1969; Thanuphasiri, 1974), visceral (Daengsvang, 1949; Miyamoto, 1994), neurological (Daengsvang, 1980; Chandenier, 2001) or a combination of these.

The most common clinical manifestation is intermittent subcutaneous migratory swelling which can appear anywhere on the body and are accompanied by pruritus and systemic symptoms such as low-grade fever, loss of appetite and nausea. Other organs such as liver, eye, lung, genitourinary, gastrointestinal tract and central nervous system may be involved (Rusnak et al., 1993). The patient may die from brain damage when the larvae move into the brain (Daengsvang, 1980).

While human gnathostomiasis cases have been reported in Lao PDR since 1975 (Fontan et al., 1975), little is known about this disease in this country. The disease is endemic in Asian countries, especially in Thailand and Japan. Recently, the endemicity of this disease has been all over the Indochina peninsula as well as Latin America (Nawa et al., 2004; Camacho et al., 2003). Sporadic cases have been reported among travelers who visited the endemic areas in Asia and Latin America. Gnathostomiasis is an important disease for travel medicine (Nawa et al., 2004). Adult worms of *G. spinigerum* are found throughout Asia in the stomachs of the definitive hosts: cats, dogs and wild carnivores such as mongoose. Natural infections with *G. spinigerum* in stray dogs and cats range from 1.1-6.9% and 1.9-8.3%, respectively and (Rojekittikhun, 2005). The northeast Thailand was reported of 4.1% of dogs was Gnathostome egg positive (Maleewong et al., 1992). From 1989-2005, about 100-400

cases of suspected human gnathostomiasis were seen at the Hospital for Tropical Disease, Bangkok, Thailand. (Rojekittikhun, 2005).

In many areas of Lao PDR, consumption of raw or improperly cooked fish and raw meat are common (Sayasone et al., 2007). Some dishes are often considered as traditional culture. During 1996-1997, two cases of gnathostomiasis were reported from Laotian women in Pakngeumg and Xaysetha district, Vientiane capital. The worms were confirmed as *G. spinigerum* in the Department of Parasitology, Faculty of Medicine, Khon Kean University (Phoumindr, 1999). Recently, a thesis study of the student in the Institut de la Francophonie pour la Médecine Tropicale (IFMT) in Vientiane capital, Lao PDR demonstrated 0.66% of the prevalence of gnathostomiasis in Paksan district, Borikhamxay province in 2005, which were diagnosed by signs, symptoms and eosinophilia > 5% (Thotsakanh, 2005). Recently, there was one case report of gnathostomiasis from Laotian woman, one week after returned from visiting to her home country. She was diagnosed by using Western blot technique (Hennies, 2006). Hence, it has been proposed that *G. spinigerum* may be responsible for human cases of gnathostomiasis in Lao PDR. In addition, the Immunodiagnostic Unit for Helminthic Infections, Department of Helminthology, Faculty of Tropical Medicine, has examined a number of serum samples of Lao patients and the patients from European countries who had a travelling history in Laos as shown in Table 1.1. (Dekumyoy, personal communication). However, there have been few published reports on gnathostoma infections in Laos. Data on the nationwide prevalence of gnathostomiasis is not available. The report of the prevalence of gnathostomiasis in the neighbor country, as Thailand, was 3.05% (Suntharasamai, 1990), Thailand and Laos has very similar tradition, culture and consumed behavior.

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Table 1.1 Immunodiagnosed gnathostomiasis data in Lao PDR

Year	Native nationality	Country living	No. of cases	Description
2003	French	France	1	traveled in Laos and +/immunoblot
2003	Laotian	France	1	+/immunoblot
2003	Laotian	France	1	presented 6 years recurrent swelling and +/immunoblot
2003	German	Germany	1	traveled in Laos/Cambodia, highly eosiniphilia, recurrent swelling and +/immunoblot
2004	Laotian	Germany	1	17% eosinophilia, swelling of knees and +/immunoblot
2006	Laotian	Canada	1	recurrent swellings of right forearm for 1 year and +/immunoblot

In Laos, many patients were clinically diagnosed as gnathostomiasis, but only a very few cases with parasitologic demonstration were included in the report or literature. Lao people had lack of the disease knowledge for clinical symptoms and prevention. The reasons may be as follows (Daengsvang, 1980):

- The worms forced themselves out to the exterior with discharge without the knowledge of the patients.
- The worms are easily thrown away accidentally by the patients.
- The worms may not be reported by the attending physician for various reasons.
- The worms after infecting human hosts might be encapsulated in the affected tissue and make no migration or eventually die in the tissue due to host reactions observed in experimental primates (Daengsvang, 1971).

For tissue nematode infections, such as gnathostomiasis, reliable diagnostic method is lacking. The diagnosis of this parasitic infection by using conventional laboratory methods, such as blood count, cerebrospiral fluid (CSF) examinations and other parasitologic approaches are not satisfactory and show doubtful results, especially in the cases of central nervous system involvement

(Bhaibulaya, 1978). It is generally based upon clinical features of meningeal cerebral symptoms with eosinophilic pleocytosis caused by *G. spinigerum*. The histories of consuming known intermediate or paratenic hosts of the parasite caught in endemic areas is helpful but not confirmatory. Diagnosis of the disease can be made by observation of migratory skin lesions, sero-positive by ELISA, immunoblot to a *G. spinigerum* antigen. Definitive diagnosis can be made by morphological identification of the larvae obtained from skin biopsies or that had migrated out spontaneously from the skin lesions, but it is less successful to get the larvae. Currently, immunoblot analysis of the patient's serum against an aqueous extract of the third-stage larvae of *G. spinigerum* for revealing antibodies specific to the parasite's specific antigen appears to be the most promising immunodiagnostic test. Gnathostomiasis has been diagnosed in an excellent evaluation of immunoblot to detection of total IgG and continued finding specificity (Dekumyoy et al., 2002; Dekumyoy, personal communication).

This study was carried out in the area located in northern, central and southern parts of Lao PDR, where the gnathostomiasis cases were reported. The results of this study showed the epidemiological feature of *Gnathostoma* and gnathostomiasis in Lao PDR.

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CHAPTER II
OBJECTIVES

2.1 General objectives

To investigate *Gnathostoma* and gnathostomiasis; and the risk factors related to gnathostomiasis in three provinces of Lao PDR.

2.2 Specific objectives

2.2.1 To detect antibody of *Gnathostoma* infection by immunoblot technique
in three provinces of Lao PDR.

2.2.2 To determine the prevalence of gnathostomiasis in three provinces of Lao PDR.

2.2.3 To describe study area on people's eating behaviour, risk factors related to the prevalence of gnathostomiasis in three province of Lao PDR.

2.2.4 To investigate potential 2nd intermediate and definitive hosts of *Gnathostoma* spp. in three provinces of Lao PDR.

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CHAPTER III
LITERATURE REVIEW

3.1 General introduction

Gnathostomiasis means the infection or disease in man or animal definitive host caused by small nematode of the genus *Gnathostoma* (Daengsvang, 1981). Human gnathostomiasis is a disease primarily cause by larva and immature stages of *G. spinigerum* (Daensavang, 1980; Miyazaki, 1991). However, four other species: *G. hispidum, G. doloresi, G. nipponicum,* and *G. binucleatum* are also known to caused disease (Araki, 1986; Ogata et al., 1988; Ando et al., 1988; Nawa et al., 1989; Almeyda-Artigas, 1991; Akahane et al., 1998). The first discovery and description on this parasite was reported by Richard Owen in 1836 from gastric tumors of a young tiger which died from rupture of the aorta in the London Zoo. Till now, 23 species of *Gnathostoma* have been published in the literature (Table 3.1). However, only 11-13 species of the genus *Gnathostoma* are considered to be valid (Daengsvang, 1980, 1982; Miyazaki, 1991; Almeyda-Artigas, 1991; Nawa et al., 2004; Rojekittikhun, 2005) (Table 3.2). The first case of human gnathostomiasis was reported by Levinsen in 1889, the worm supplied by Dr. Deuntzer who obtained from a tumor swelling in the breast of young Thai woman living in Bangkok (Levinsen, 1889; Daengsvang, 1980; Daengsvang, 1983; Feinstein, 1984). The immature female worm identified by Levinsen as *Cheiracanthus siamensis* n. sp. was later proven to be synonymous with *G. spinigerum*.

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Table 3.1 The first records of *Gnathostoma* spp. in the literature up to 1991

	Species	Natural definitive hosts	Habitat	Locality
1.	***G. spinigerum*** Owen, 1836	*Felis tigris (tiger)*	Stomach wall	England
2.	***G. robustum*** Diesing, 1838	*Felis concolor*	Stomach wall	Brazil
3.	***G. gracile*** Diesing, 1838	*Arapaima gigas* (fish)	Intestinal canal	Brazil
4.	***G. horridum*** Leidy, 1856	*Alligator mississippiensis*	Stomach lumen	USA
5.	***G. sociale*** Leidy, 1856	*Mustela vison* (mink)	Stomach wall	USA
6.	***G. radulum*** Schneider, 1866	*Paradoxurus philippinensis*	Stomach wall	Philippines
7.	***G. hispidum*** Fedtschenko, 1872	*Sus scrofa ferus* & *Sus s. domesticus* (pig)	Stomach wall	Turkestan & Hungary
8.	***G. pelecani*** Chatin, 1874	*Pelecanus onocrotalus*	Subcutis (thorax) & air sac	Europe
9.	***G. siamensis*** Levinsen, 1889	*Homo sapiens* (human)	Subcutis	Thailand
10.	***G. trugidum*** Stossich, 1902	*Didelphis azarae* (opossum)	Stomach wall	Argentina
11.	***G. paronai*** Porta, 1908	*Rattus rajah* (rat)	Intestinal canal	Indonesia
12.	***G. accipitri*** Skrjabin, 1951	Eagle	Subcutis (head)	Turkistan
13.	***G. americanum*** Travassos, 1925	*Felis tigrina*	Stomach wall	Brazil
14.	***G. doloresi*** Tubangui, 1925	*Sus scrofa domesticus*	Stomach wall	Philippines
15.	***G. didelphis*** Chandler, 1932	*Didelphis virginiana*	Liver	USA
16.	***G. nipponicum*** Yamaguti, 1941	*Mustela sibirica itatsi* (weasel)	Esophageal wall	Japan
17.	***G. procyonis*** Chandler, 1942	*Procyon lotor lotor* (raccoon)	Stomach wall	USA
18.	***G. minutum*** Stekhoven, 1943	Serpent	Connective tissue	Congo
19.	***G. brazilianse*** Ruiz, 1952	*Lutreolina crssicaudata*	Liver	Brazil
20.	***G. miyazakii*** Anderson, 1964	*Lutra c. Canadensis* (otter)	Kidney	Canada
21.	***G. vietnamicum*** Le Van Hoa, 1965	*Lutra elioti* (otter)	Kidney	Vietnam
22.	***G. malaysiae*** Miyazaki & Dunn, 1965	*Rattus surifer* (rat)	Stomach wall	Malaysia
23.	***G. binucleatum*** Almeyda-Artigas, 1991	*Felis p. pardalis* (ocelot)	Stomach wall	Mexico

(Modified from Table 2.6, Waikagul et al., 2003)

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The present world, with the expansion of international travel and the increase of immigration, the number of persons in Europe and other Western countries that have returned from foreign travel in areas of parasitic infections or have immigrated with infections rarely seen previously has been increasing. Among those diseases caused by helminthic parasites is gnathostomiasis, a disease once confined primarily to Southeast Asia. Physicians now must be more alert to the symptoms and treatments available (Ligon, 2005).

Table 3.2 Currently accepted *Gnathostoma* species with reference to human infection

	Species	Natural definitive hosts	Habitat	Locality	Human infection
1.	***G. spinigerum*** Owen, 1836	Feline, canine	Stomach	Asia, Oceania	Yes
2.	***G. hispidum*** Fedtschenko, 1872	Pig, wild pig	Stomach	Asia, Europe	Yes
3.	***G. trugidum*** Stossich, 1902	Opossum	Stomach	Americas	
4.	***G. americanum*** Travassos, 1925	Feline	Stomach	South America	
5.	***G. doloresi*** Tubangui, 1925	Pig, wild pig	Stomach	Asia, Oceania	Yes
6.	***G. didelphis*** Chandler, 1932	Opossum	Liver	USA	
7.	***G. nipponicum*** Yamaguti, 1941	Weasel	Esophagus	Japan	Yes
8.	***G. procyonis*** Chandler, 1942	Raccoon	Stomach	USA	
9.	***G. brazilianse*** Ruiz, 1952	Otter	Liver	Brazil	
10.	***G. miyazakii*** Anderson, 1964	Otter	Kidney	North America	
11.	***G. malaysiae*** Miyazaki & Dunn, 1965	Rat	Stomach	Malaysia, Thailand	
12.	***G. vietnamicum*** Le Van Hoa, 1965	Otter	Kidney	Vietnam, Thailand	
13.	***G. binucleatum*** Almeyda-Artigas, 1991	Canine	Stomach	Mexico	Yes

(Modified from Table 1, Nawa et al., 2004)

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3.2 History and geographical distribution

3.2.1 *Gnathostoma spinigerum*

This species is the most important human pathogen in the genus of *Gnathostoma*. This nematode was first discovered in a tumor on the gastric wall of a tiger which died in the London Zoo, was described and named as *G. spinigerum* by Richard Owen in 1836. Half a century later, a worm of this species was found for the first time in human being from the chest skin of a young Thai woman living in Bangkok and given a name of *Cheiracanthus siamensis* by Levinsen, 1889 (now a synonym of *G. spinigerum*). It has been particularly prevalent in Thailand since long ago, and known as 'tua-chid'(meaning painful tumor or piercing pain) (Daengsvang, 1983; Miyazaki, 1991; Rojekittikhun, 2005). It was distributed mainly in Asia including Palestine, India, Bangladesh, Myanmar, Thailand, Lao PDR, Cambodia, Vietnam, Malaysia, Indonesia, the Philippines, China, and Japan while also present in Australia and the American Continent (Rusnak et al., 1993; Miyazaki, 1991). It was widespread in Japan in 1955-1957, but the prevalence declined afterward mainly because of changes in eating behavior (Miyazaki, 1991).

3.2.2 *Gnathostoma hispidum*

It was first found in the gastric wall of wild pigs in the Turkestan district and swine in Hungary (Fedtschenko in 1872). Infection among this animal was commonly found in Europe and Asia. Human infection was almost limited in Japan. First case was reported in Japan with lesions of the skin (Morishita, 1924) and and second case was reported in China with lesion in the eye (Chen, 1949). Nevertheless, Miyazaki (1960) was very skeptical about the species identification of the above two reports (Daengsvang, 1980). During 1980-1986, more than 50 human cases that had probably been caused by this parasite were reported in Japan. Most cases have a history of eating loaches, *Misgurnus anguillicaudatus*, imported from Taiwan, China or Korea (Nawa et al., 1989; Kagei, 1991; Taniguchi et al., 1992). Till now, three confirmed cases of human infection were also published in Japan (Akahane et al., 1998).

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3.2.3 *Gnathostoma doloresi*

This gnathostome species was first found by Tubangui in 1925 in the gastric wall of a pig in Luzon Island, the Philippines, but the description was based only upon four female worms. In 1930, Maplestone described males from pig stomachs in India. The parasite is distributed in several countries in Asia (Daengsvang, 1980; Miyazaki, 1991). Human infection were confined only in Japan, about 30 human cases were reported from the southern part of Japan (Ogata et al., 1988; Nawa, 1991; Nawa et al., 1989, 1997; Akahane, 1998; Kurokawa et al., 1998). Till now, 45 human cases due to *G. doloresi* were reported in Japan (Ando, 2005).

3.2.4 *Gnathostoma nipponicum*

This species of *Gnathostoma* was first found from the esophagus of weasels in Japan and was considered to be *G. spinigerum* by Yoshida in 1931. Later, it was identified as a new species, namely *G. nipponicum* by Yamaguti in 1941. Animal infection is widespread exclusively in Japan (Miyazaki, 1991). Recently, eight human cases were reported in Japan (Ando et al., 1988, 1991; Sato et al., 1992).

3.2.5 *Gnathostoma binucleatum*

The species of *G. binucleatum* was first described as the newest species of the genus *Gnathostoma* by Almeyda-Artigas in 1991. The specific name refers to the dominance of binuclear intestinal cells of the advanced third-stage larvae. In America, the first human case of gnathostomiasis was recorded in Mexico in 1970, though the infection had been discovered as early as 1964 in this country. The disease was found common in inhabitants in the lower Papaloapan River basin, Veracruz, in Mexico (Almeyda-Artigas, 1991), and more than 1,000 people in six endemic areas were reported to be infected with the parasite during 1980-1996. However, whether the parasite responsible for gnathostomiasis in Mexico, is in fact a novel species or a geographical isolate of a known *Gnathostoma* species, it is now under investigation by several researchers (Camacho et al., 1998; Koga et al., 1998; Ogata et al., 1998; Rojas-Molina et al., 1999). The validity of *G. binucleatum* and its role in human gnathostomosis in Mexico and Ecuador is currently elucidated by Almeyda-Artigas and colleagues in 2000.

3.3 Classification of the parasite

Phylum	:	Nemathelminthes	
Class	:	Nematoda	(Rudolphi, 1808)
Subclass	:	Secernentea	(Dougherty, 1958)
Order	:	Spirurida	(Chitwood, 1933)
Suborder	:	Spirurina	(Railliet & Henry, 1913)
Superfamily	:	Gnathostomatoidea	(Lane, 1923)
Family	:	Gnathostomatidea	(Blanchard, 1895)
Subfamily	:	Gnathostomatinae	(Blanchard, 1895)
Genus	:	*Gnathostoma*	(Owen, 1836)

3.4 Morphology

Adult *Gnathostoma* usually are approximately 2 to 3 cm in length and are rust-colored. The worms have a "mouth" and an anus (Figure 3.1). Nutrients are ingested through two lips that are located at the tip of the cephalic bulb (Figure 3.2). The digestive tract is not complex, comprising merely an esophagus and an intestine. Two types of papillae, a cervical papilla off the main body and two labial papillae on the cephalic bulb, extend from the worm. The larvae range in size from approximately 2 mm to more than 16 mm. *Gnathostoma* larvae usually have four rows of hooklets that extrude from the surface of the cephalic bulb; the rows are used to help the worm lodge into the tissues of the hosts. Tiny, cuticular spines run along the length of their bodies (Figures 3.3-3.6). From the medical standpoint, *G. spinigerum* is considered to be the most important species. Hence, only *G. spinigerum* is described in detail and other species are described in brief.

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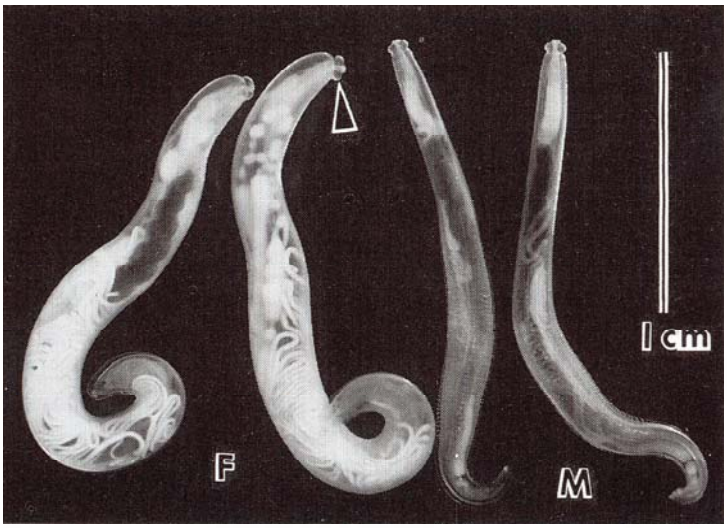

Figure 3.1 *G. spinigerum*: females (F) and males (M). Arrow: head-bulb. White esophagus, black midgut, and white genital organs are clearly seen especially in the female body. (From Miyazaki I, Fukuoka Acta Med., 52(25), 1961)

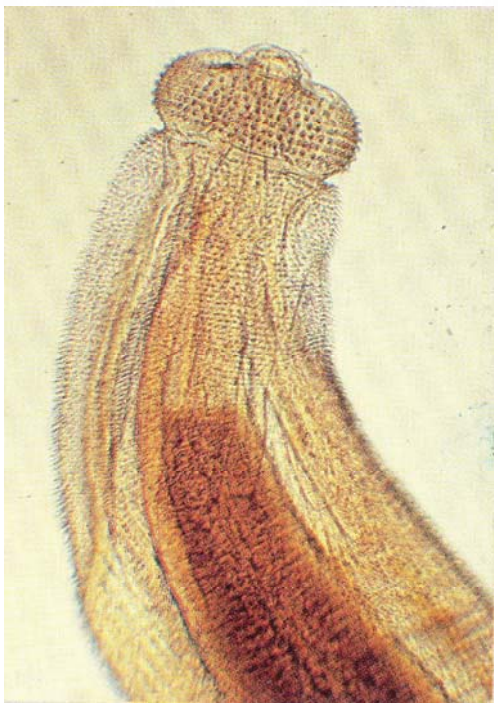

Figure 3.2 *G. spinigerum* adult, with a cephalic bulb armed with 8 rows of hooks. There are two lips surrounding the mouth. (From Radomyos *et al*, 2004)

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Figure 3.3 Cuticular spines of the posterior body part. *G. spinigerum* from cats in Laos (Scholtz *et al,*1990) Used with permission of CAB International. Available at http:www.dpd.cdc.gov/DPDx/HTML/ImageLibrary.htm

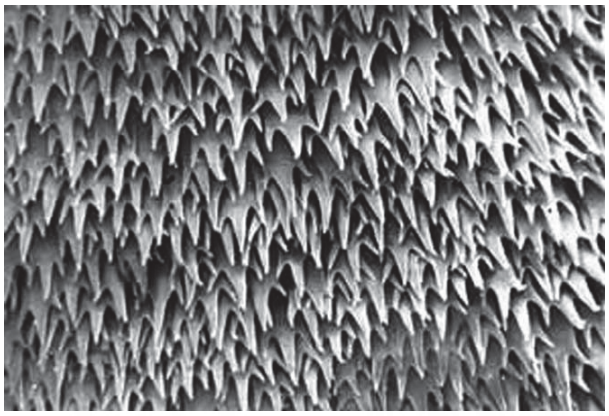

Figure 3.4 Details of cuticular spines of the anterior body part. *G. spinigerum* from cats in Laos. (Scholtz *et al,*1990) Used with permission of CAB International. Available at http:www.dpd.cdc.gov/DPDx/HTML/ImageLibrary.htm

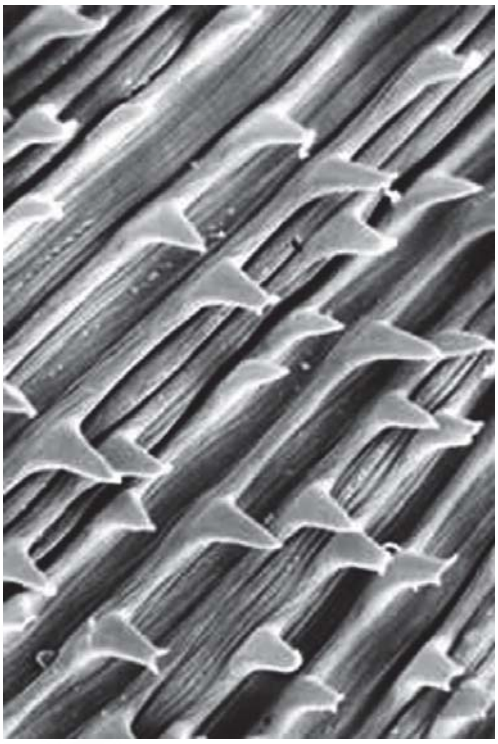

Figure 3.5 Detail of nondendiculated cuticular spines. *G. spinigerum* from cats in Laos.
(Scholtz *et al,*1990) Used with permission of CAB International. Available at http:www.dpd.cdc.gov/DPDx/HTML/ImageLibrary.htm

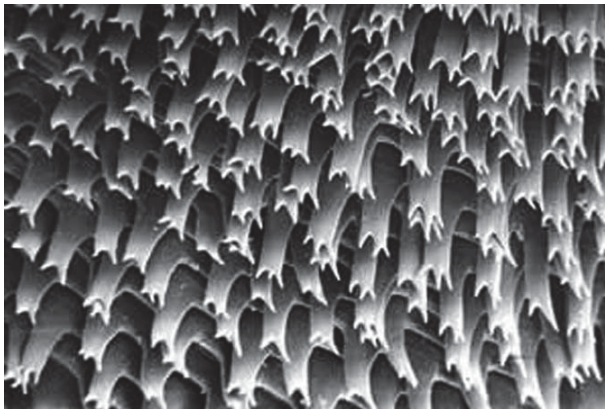

Figure 3.6 Details of cuticular spines of the anterior body part. *G. spinigerum* from cats in Laos. (Scholtz *et al,*1990) Used with permission of CAB International. Available at http:www.dpd.cdc.gov/DPDx/HTML/ImageLibrary.htm

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3.4.1 *G. spinigerum*

3.4.1.1 Adult

The adult worm is cylindrical with a globular head or head-bulb and has a thick and short body, in general whether male or female, is provided with at least 7-9 cephalic hooklet rows. These are about equal in size, 13 μm long x 8 μm wide, gradually increasing in number from row one close to the lips till row 8 next to the neck. Each cephalic hooklet has an oblong base (Figures 3.7; 3.8; 3.9). The shape and size of the cuticular spines vary, according to their locations on the adult gnathostome body. The numbers of cuticular spines gradually reduce in size and density posteriorly and become smaller and shorter with decreasing number of teeth. The spines become single-pointed end as they approach the posterior third of the body. From this junction downwards, the cuticle is observed to be naked except for scarce remnant-like spines irregularly arranged in different areas (Figures 3.9-11) (Daengsvang, 1980; Miyazaki, 1991).

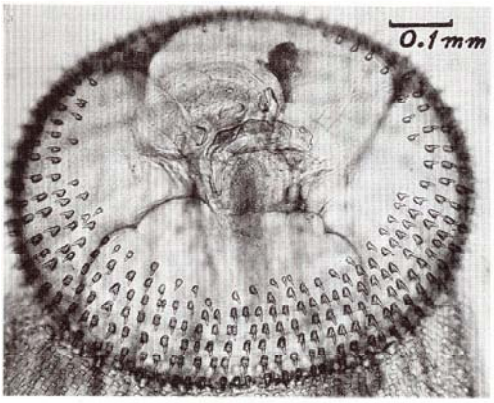

Figure 3.7 *G. spinigerum*: Enlarged head-bulb. A pair of lips in the center and four ballonets around them. Generally, eight rows of hooks are aligned. (From Miyazaki I, Fukuoka *Acta Med*., 52(25), 1961)

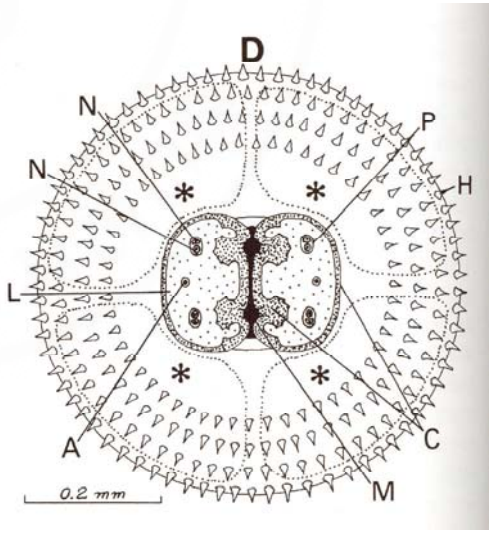

Figure 3.8 *G. spinigerum*: Anterior view of the swollen head-bulb. D: dorsal side, A: amphid, C: cuticle of lips (L), H: head-bulb hooks, M: mouth, N: end of nerve, P: sensory papilla, * four ballonets. (From Takeichi T., *Acta Med*., 26(10), 1956)

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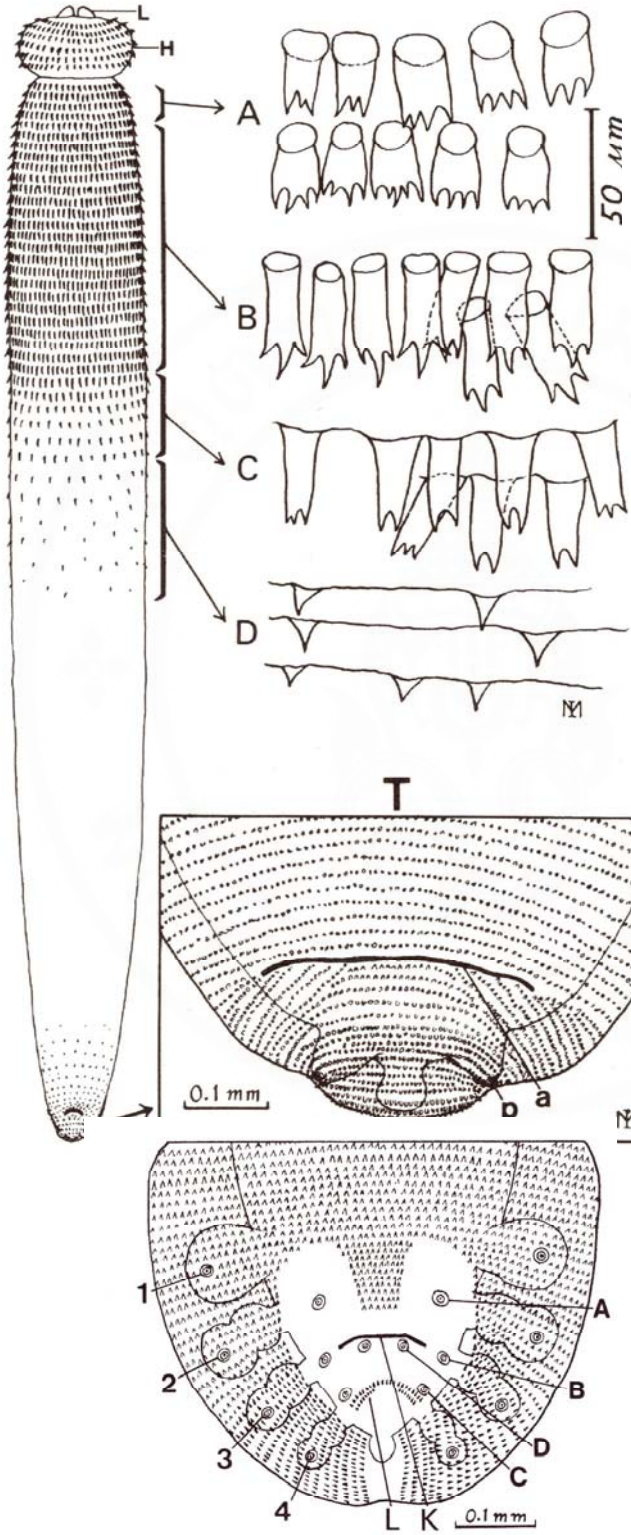

Figure 3.9 *G. spinigerum*: Body surface of the female. Anterior half of the body is covered with cuticular spines, of which B-type is typical and abundant, Posterior half of the body is naked, except the tail (T). a: anus, p: papilla, L: lips, H: head-bulb (From Miyazaki I, 1960)

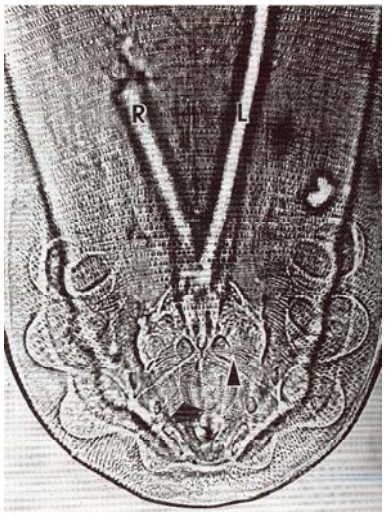

Figure 3.10 *G. spinigerum*: Tail end of a male in ventral view. Numerous small cuticular spines grow forward. L: posterior half of left spicule, R: right spicule, Triangle: cloacal aperture, Arrow: spinelets grown in arc. (From Miyazaki I, 1990)

Figure 3.11 *G. spinigerum*: Ventral view of the caudal ala of a male (spicules omitted). 1-4: pendunculate papillae, A-D: small papillae, K: cloacal aperture, L: spinelets arranged in arc. (From Takeichi, T., Acta Med.,26(10), 1956)

3.4.1.2 Male

Generally, the distinguishing features of the male *G. spinigerum* are the following: spicules unequal, presence of four pairs of large lateral, and two pairs of small ventral, caudal papillae. Cuticular spines cover the body anteriorly, about half or two-thirds of the lengths, leaving the lower part of the body naked except on the greater part of the posterior 0.8mm of the ventral surface of caudal extremity, where there are closely set minute spines. There is a naked Y-shaped area around the cloacal aperture behind which a few minute spines are seen arranged in short arched lines (Figures 3.10; 3.11). The tail of the male worm is red and curled to the ventral side (Miyazaki, 1960, 1991; Daengsvang, 1980; Rojekittikhun et al., 1998). The average size is 26.2 (16-40) mm long and 1.8 (1-3) mm wide, and for the head-bulb, the average is 0.4 (0.2-0.6) mm long and 0.6 (0.5-0.9) mm wide (Daengsvang, 1980).

3.4.1.3 Female

The characteristic of the adult female closely parallels with that of the adult male, but in comparison at the same age, female are always larger than males (Miyazaki, 1991). Essentially, the following are noted down as bases for identification: there is a slight transverse slit vulva behind the middle of the body, opening into a long muscular vagina running anteriorly, then posteriorly and dorsally before dividing to two uterine branches. In the vagina, eggs are seen with thin colorless shell, with marked thinning at one pole before a cap-like knobbed end. The average size of the female is 34.7 (13-55) mm long and 2.3 (1-3) mm wide, and for the head-bulb, the average size is 0.56 (0.4-0.7) mm long and 0.97 (0.7-1.2) mm wide. Laterally, the tail is rounded dorsally and flattened ventrally, while on the ventral view, its end is bluntly rounded and carries close to the tip a pair of unusually large caudal papillae (Daengsvang, 1980). At the tail end of the female worm, small spines are aligned on the ventral surface, densely arranged in many transverse rows (Miyazaki, 1991). This feature distinguishes *G. spinigerum* and *G. nipponicum*, which has no visible spines on its terminal end.

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3.3.1.5 Larvae

G. spinigerum has several larval stages in the life cycle; after around 12 days in fresh water 21-31˚C, the newly laid eggs from the female worm could be hatched become first-stage larvae which are free living actively motile larvae, are cylindrical enveloped in a delicate, voluminous smooth and transparent sheath much similar to microfilaria sheath. The sheath and cuticle are smooth, with no striations, except for wrinkles at places where larva bends (Daengsvang, 1980). The average size, excluding sheath is 265.2 x 15.8 μm while the average size for the ensheathed larva is 300.5 μm long (Prommas et al., 1933). Miyazaki (1960) recorded the ensheathed larva to be nearly 0.3 mm long.

Then, after being ingested by the cyclops, the first-stage larva becomes unsheathed in the digestive tract of the cyclops and migrates to the hemocoel to become a complete second-stage larva (Daengsvang, 1980; Miyazaki, 1991). The average size at this period is 296 x 13 μm.

After the second molt in the cyclops it becomes an early third-stage larva, and already has characteristics of *Gnathostoma* (Miyazaki, 1991). It much increases in size to the average of 460 x 45 μm. The head-bulb is clearly seen bearing four rows of hooklets each row bearing slightly more than 40 minute hooklets measuring about 2 x 3 μm (Daengsvang, 1980). The average numbers of the hooklets are 43.2 in the first row, 44.8 in the second, 46.7 in the third and 52.3 in the fourth. The 4 ballonet-cervical sac systems are seen (Miyazaki, 1991). The larva shows no further change in morphology having developed to this stage in cyclops (Figure 3.12; 3.13). The advanced third-stage larva will can be developed further while infected cyclops with early third-stage larva are ingested by second intermediate hosts. The advanced third-stage larva is considered as infective larval stage to definitive hosts and also human.

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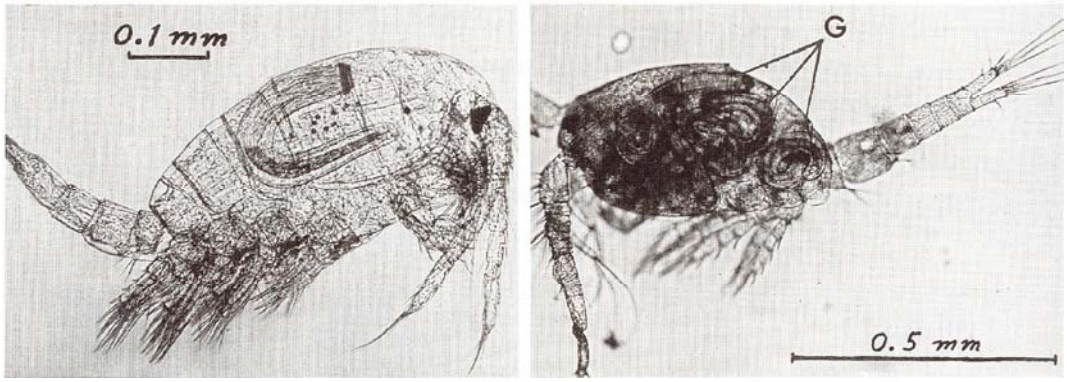

Figure 3.12 *G. spinigerum*: Early third-stage larvae parasitic in the hemocoel of cyclops. A larva in the left and several ones (G) in the right (From Miyazaki I, 1991).

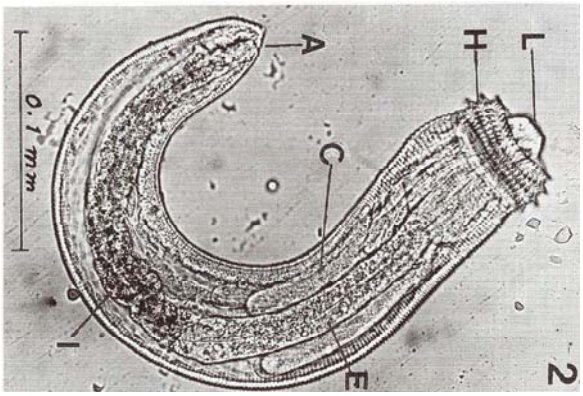

Figure 3.13 *G. spinigerum*: Early third-stage larva removed from the host. L: lip, H: headbulb, C: cervical sac, E: esophagus, I: intestine, A: anus.
(From Miyazaki I, 1991).

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3.4.1.5 Advanced third-stage larva

This larval stage is more important than others, found in the second intermediate, paratenic hosts and human gnathostomiasis. The larva can be use as the stage for differentiates to species of *Gnathostoma*. When the infected cyclops with early third-stage larva were eaten by the second intermediate hosts, the larva increases in size, become the advanced third-stage larva, which coils around its own head end and enveloped by thin cyst (Daengsvang, 1980; Miyazaki, 1991). The cyst is round in shape, slightly yellowish gray in color with the diameter of about 1.14 mm. The cyst wall is thin, semi-translucent and through it one can see a coil of one larva (Figure 3.14).

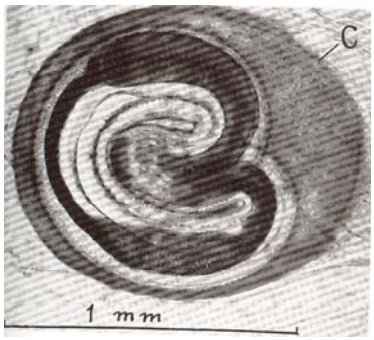

Figure 3.14 *G. spinigerum*: Advanced third-stage larva encysted in the muscle of a fish, *Channa argus*. The cyst (C) is connective tissue made by the host; it contains a little turbid liquid. (From Miyazaki I., *Exp. Parasit.,* 9(3), 1960).

After dissection from the cyst wall, the advanced third-stage larva is morphologically divided into 3 different parts, lips, head-bulb or cephalic bulb, and body with constriction or neck between head-bulb and body. The average size of the advanced third-stage larva is 3.95 x 0.42 mm (ranging 2.8-5.2 x 0.3-0.8 mm) (Daengsvang, 1980). In the middle of the head-bulb has a pair of lips, which are protruded as in the adult (Miyazaki, 1991). The average size of head-bulb is 0.16 mm long and 0.28 mm wide (Daengsvang, 1980). On the head-bulb, hooklets are aligned in 4 transverse rows of well-developed single-pointed hooklets (Daengsvang, 1980; Miyazaki, 19991). The hooklets are practically the same size and structure except that

those of row 1 beginning from the top of the head-bulb are somewhat smaller than the others, averaging 13.0 x 6.6 μm and 16.3 x 8.0 μm, respectively. The head-bulbs usually have 40 or more cephalic hooklets in each row (Daengsvang, 1980; Miyazaki, 1991). From the 69 advanced third-stage larvae mentioned above, there were some larvae had the number of cephalic hooklets less than 40, such that row 1 of 17 larvae (24%), row 2 of 3 larvae (4%), row 3 of 1 larva and row 4 of 2 larvae (3%). There was only one larva aligning 36 to 38 cephalic hooklets in each of 4 cephalic hooklet rows. However, the number of cephalic hooklets increases posteriorly from row 1 to 4 averaging 41.5, 43.3, 46.6, 49.2, respectively (Daengsvang, 1980) whereas Miyazaki reported that 44.3, 47.3, 49.6, and 52.0, respectively. Shape and number of the cephalic hooklets are useful for species identification (Figure 3.15). Counting the number of cephalic hooklets, it is better to cut the head-bulb off the body and have a front view to observe (Miyazaki, 1960; 1991).

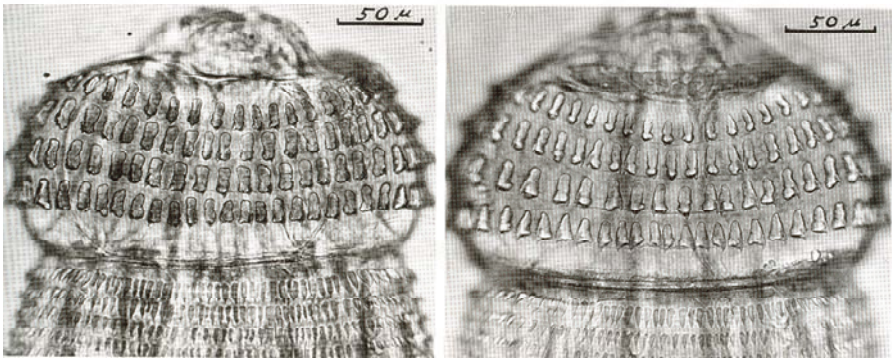

Figure 3.15 *G. spinigerum*: Headbulb of advanced third-stage larva. Hooks are arranged in four rows and are slightly smaller in the 1st and the 4th rows. (From Miyazaki I., Kyushu Mem. Med. Sci., 5(2), 1954).

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The whole body surface is transversely striated and covered with about 200 transverse rows of single-pointed cuticular spines down to the tail end, each cuticular spine measuring about 8 μm long at the anterior part of the body and about 2-3 μm long at its posterior part. The cuticular spines are numerous on the anterior half of the body and become gradually less in number and size toward the posterior extremity (Daengsvang, 1980). Miyazaki 1991 found that the total number of rings of spines is always more than 200, which are about 10 μm in length and progressive smaller towards the posterior end, where they become only about 2 μm in length (Figure 3.16) (Miyazzaki, 1991). The prior investigators reported the absence of these spines on the posterior half of the body (Chandler, 1925b; Prommas et al., 1936; Daengsvang et al., 1938).

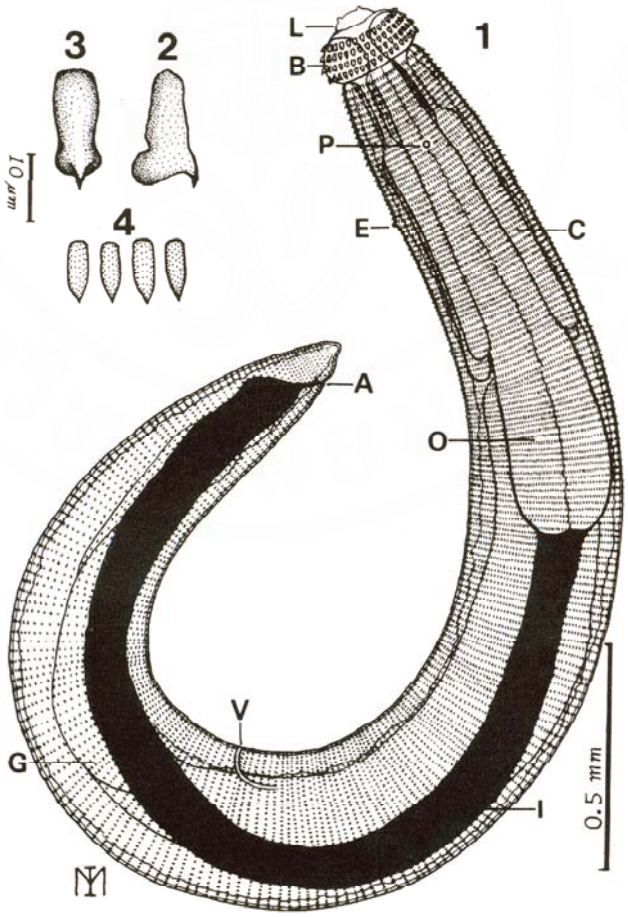

Figure 3.16 *G. spinigerum*: Structure of advanced third-stage larva. **1**: Lateral view of the body, L: lip, B: ballonet, P: cervical papilla, E: excretory pore, C: cervical sac, O: esophagus, I: midgut, V: vulva, G: lateral line, A: anus. **2**: Lateral view of the headbulb hook, free side at right. **3**: Dorsal view of the same hook. **4**: Spines on the anterior part of the body, which become smaller posteriorly. The rows of body spines amount over 200.
(From Miyazaki I, 1960).

As the internal structure of the larva, 4 globular sacs or ballonet-cervical sac systems are seen in the head-bulb. In the living larva, the cervical sacs are seen continually moving in irregular manner (Daengsvang, 1980; Miyazaki, 1991). The esophagus is large and long with a well-developed esophageal gland. The male and female worms of the advanced third-stage larva can be distinguished by observation of the presence of the vulva, which indicates the female sex. Diagnosis of male worm is rather difficult, but a close examination of the tail end will reveal an ejaculatory duct and spicules, which are still rudimentary (Miyazaki, 1991).

3.4.1.6 Immature adult

The worm has already 7-8 rows of cephalic hooklets (Daengsvang, 1975; 1980). It is noted that the beginning of reproductive organ differentiation is evident at this point, in contrast to the genital primordium found in the larva stage. The cuticle bears rows of transversely arranged cuticular spines, at first dense anteriorly and becoming smaller and sparsely situated posteriorly. The first rows from the neck are 4-5 toothed. The succeeding rows bear three-toothed spines, becoming single-toothed posteriorly at first dense at the middle half, and later, sparsely situated at the posterior half appearing with the exception of the ventral surface of the male caudal extremity, where there are minute spines (Miyazaki, 1960). This stage are similar the adult worm, except for the undifferentiated reproductive organs.

3.4.1.7 Egg

It has ovoid-shape and a prominent transparent knob at one end. It is brownish and thin shelled, the shell having a fine granulation on the surface. The average sizes of eggs, which include knob, are 68.7 x 36.7 μm (Prommas et al., 1933) and 69.3 x 38.5 μm (Miyazaki, 1960). Therefore, the size of the eggs varies from 55.9-79 x 34.6-42.6 μm (Daengsvang, 1980). In fresh stools of the final host, the eggs are mostly unsegmented or may contain two to four cells (Figure 3.17). After 12 days in fresh water at room temperature, they become first-stage larvae. The motile-coiled embryos inside the eggshell hatch spontaneously usually through the knob to become free-swimming actively motile larvae (Figure 3.18) (Daengsvang, 1980).

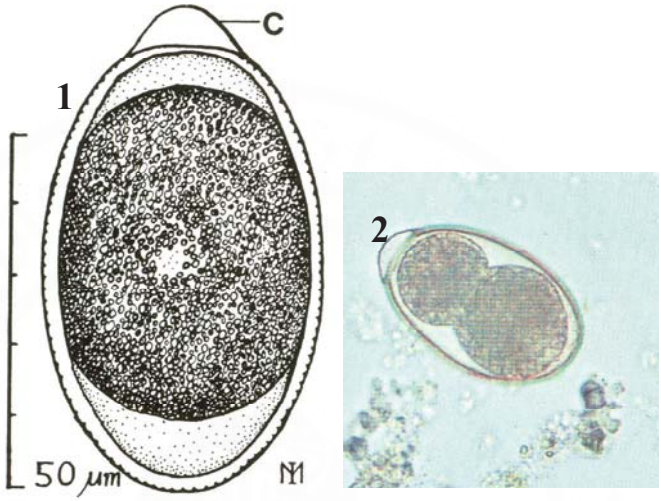

Figure 3.17 *G. spinigerum*: **1**: Fertilized egg containing an unsegmented ovum. It has a thin bulge (C) at one pole. (From Miyazaki I, 1991).
2: *G. spinigerum* fertilized egg with 2 cells.
(From Radomyos et al,. Atlas of Medical Parasitology,. 2004:102-5.)

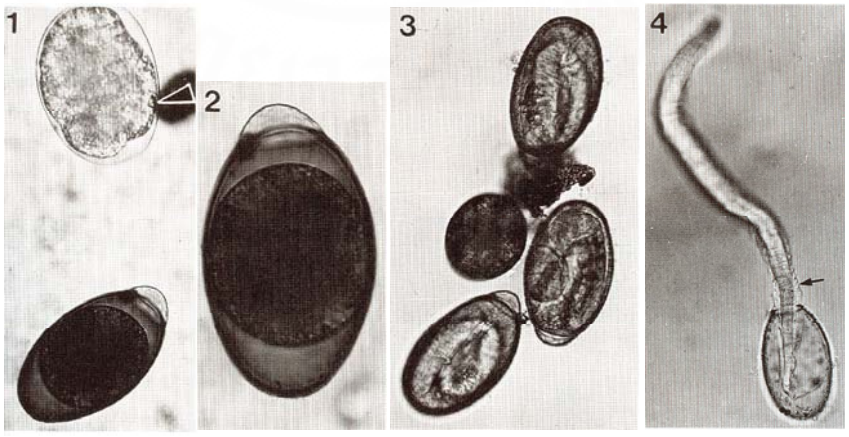

Figure 3.18 *G. spinigerum*: Development of fertilized eggs. **1**: Eggs in stool of a tiger (arrow: hookworm egg). **2**: Enlarged eeg in unicellular stage, yellow-brown. **3**: Larvae mature in a week at 27°C. **4**: Ensheathed 2nd-stage larva hatching with exuviae (arrow). (From Miyazaki I., Acta Med., 22(11), 1952.)

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3.5 Other *Gnathostoma* species proven to cause human infections

3.5.1 *Gnathostoma hispidum*

Adults of this worm are also parasitic in the gastric wall of pigs and wild boars; the body is about 2.5 cm long in female and about 2 cm long in male. The body surface is markedly different from that of the *G. spinigerum*, the whole body being covered with cuticular spines of different size and shape (Miyazaki, 1991). Daengsvang reported that the average sizes of female measures 26.2 x 2.3 mm while male are 19.7 x 1.7 mm. The head-bulbs bear 9-12 rows of cephalic hooklets (Figure 3.19; 3.20). Fertilized eggs are 65-69 x 36-40 μm with a plug at one pole, and cannot be differentiated from those of *G. spinigerum* (Figure 3.21). First-stage larvae are generally similar to those of *G. spinigerum*.

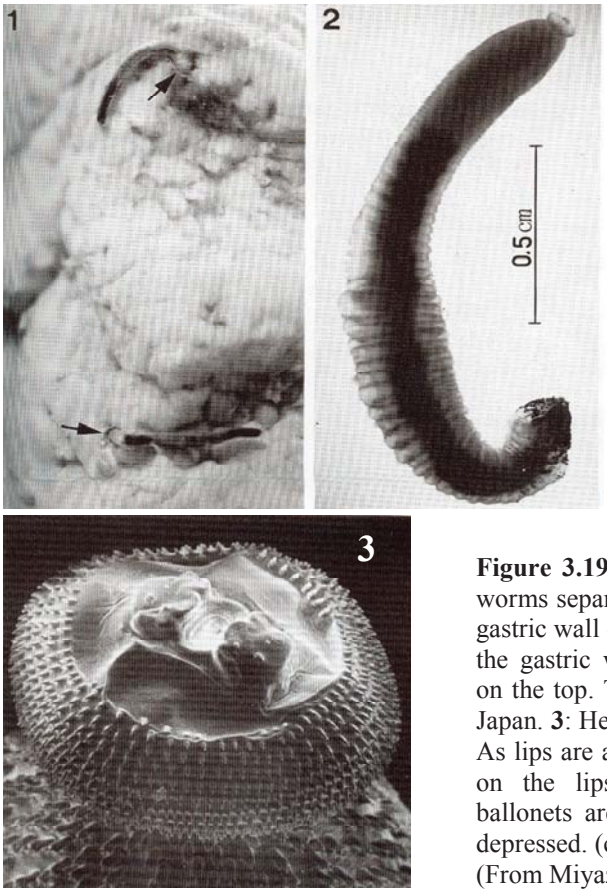

Figure 3.19 Adult of *G. hispidum*: **1**: Two worms separately in two pores (arrow) of the gastric wall of a pig. **2**: A male removed from the gastric wall, its headbulb being swellen on the top. This is the first adult obtained in Japan. **3**: Headbulb of the adult. SEM (x100). As lips are apart, the mouth is open. Papillae on the lips are shown clearly. Because ballonets are contracted, the upper plane is depressed. (courtesy of Dr. M. Koga)
(From Miyazaki I, 1991).

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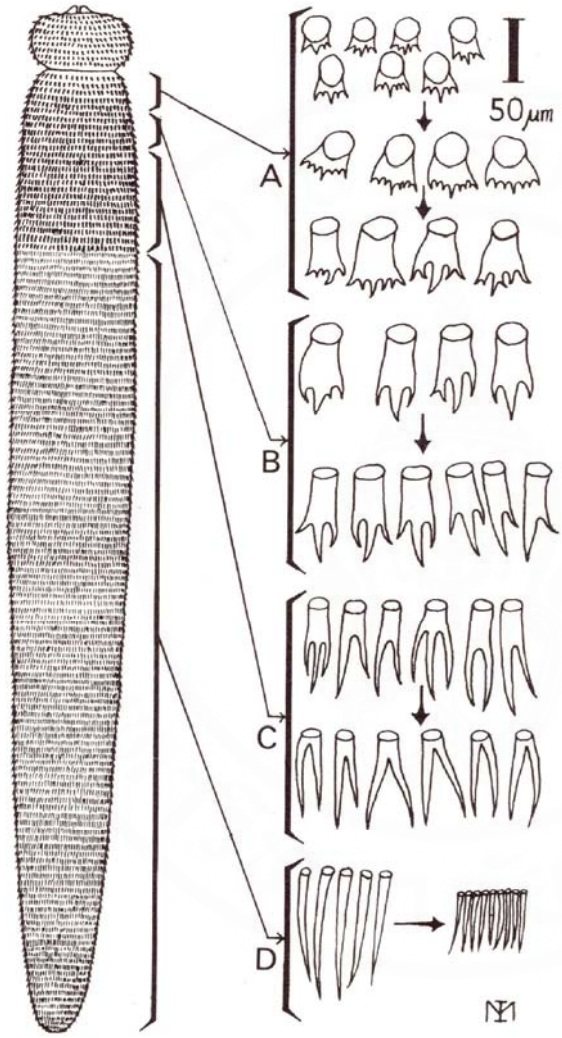

Figure 3.20
G. hispidum: Extent and shape of cuticular spines of a female collected from a pig in China.
(From Miyazaki I, 1960)

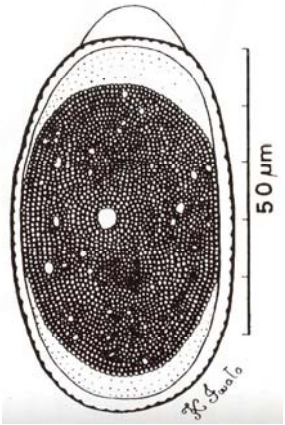

Figure 3.21 *G. hispidum*: Fertilized egg containing an unsegmented ovum; yellowish brown, with a plug at one pole.
(From Miyazaki I, 1991).

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Advanced third-stage larva is pinkish white in color. The average size, measuring 2.12 x 0.28 mm (ranging from 1.2-3.5 x 0.2-0.3 mm), the head-bulb has four rows of rectangular cephalic hooklets of which the average number from rows one to four are 38.3, 40.5, 41.8 and 46.0, respectively (Figure 3.22). The number of cephalic hooklets gradually increases posteriorly. The whole larval body is covered with many transverse rows of single-pointed spine directed posteriorly. They become gradually reduced in size and density towards the posterior extremity. (Daengsvang, 1980; Akahane et al., 1982; Koga et al., 1984, 1988; Huang et al., 1986; Akahane & Mako, 1987).

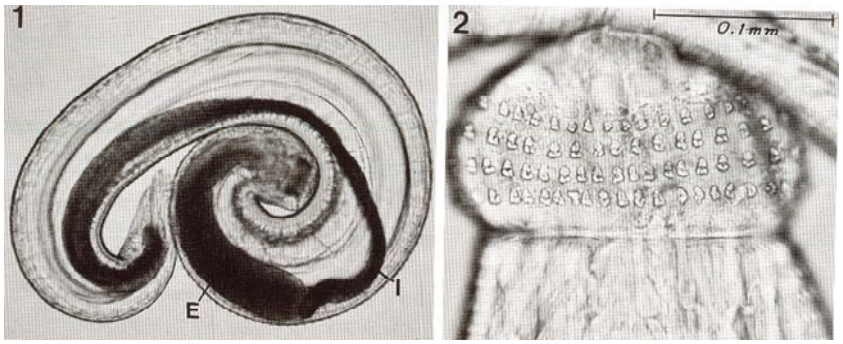

Figure 3.22 *G. hispidum*: Advanced 3rd-stage larva. **1**: Removed from the muscle of a rat; body about 3mm long, the headbulb contracted. E: esophagus, I: intestine. **2**: Headbulb; shape of hooks are similar to those of *G. doloresi*, except that those in the last row are smaller. (From Miyazaki I, 1991).

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3.5.2 *Gnathostoma doloresi*

The adult worm is similar to that *G. hispidum* in both morphology and the manner of parasitization. Single or in groups of a few, the worm inserts the anterior part of its body into the thickened gastric wall. The head-bulb is armed with 7-12 rows of backward-curving cephalic hooklets. The whole body is covered with cuticular spines of different size and shape. Females measure 8-63 x 0.9-4.5 mm while males are 7-38 x 0.9-3 mm (Figure 3.23). Fertilized eggs are 56-62 x 31-35 μm with two plugs, one plug at both poles and have a light yellow-brown color when present in stool (Figure 3.24). They have very fine pits on their rather thick shells, and are the smallest among those of all *Gnathostoma* species. First-stage larvae measure 213-306 μm. The morphology and activity are similar to those of *G. spinigerum* and *G. hispidum*.

The average sizes of the advanced third-stage larvae are measured at 2.85 x 0.38 mm, ranging from 1.83 -3.99 x 0.26-0.49 mm. The unencysted larvae were found in the liver of the two experimented mice (Daengsvang, 1980). The encysted larvae were mostly found in fleshes or sometimes in the viscera of the host and measured about 1 mm in diameter (Miyazaki, 1991). The head-bulb is aligned with 4 rows of roundish or square cephalic hooklets of which the average number from rows one to four are 35.7, 35.7, 33.4 and 33.8, respectively. Each row normally has less than 40 hooklets; the number of hooklet in the fourth row is smaller than in the first row. The each hooklets have a roundish or irregularly square base and are conspicuously small in size in the first row. These features are useful to differentiate *G. doloresi* from other *Gnathostoma* spp. (Daengsvang, 1980; Miyazaki, 1991).

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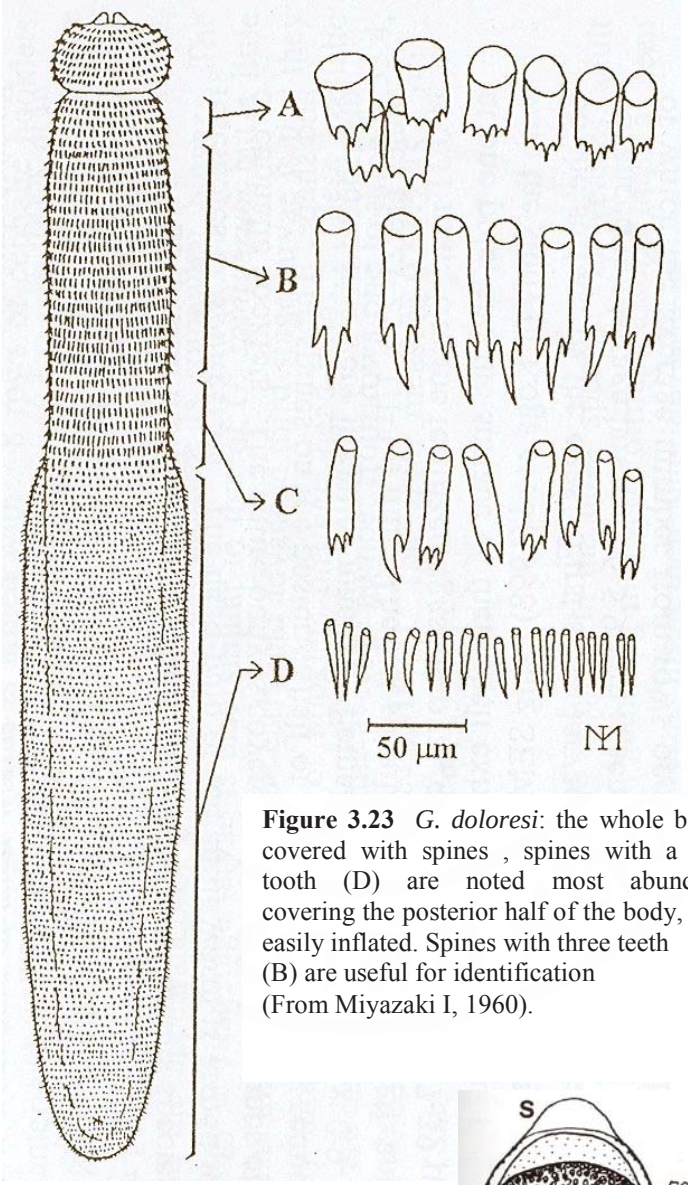

Figure 3.23 *G. doloresi*: the whole body is covered with spines , spines with a single tooth (D) are noted most abundantly, covering the posterior half of the body, that is easily inflated. Spines with three teeth (B) are useful for identification (From Miyazaki I, 1960).

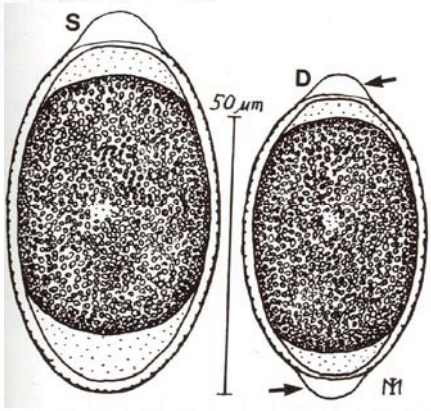

Figure 3.24 *G. doloresi*: Fertilized egg (D) with bulges (arrow) at both poles, *G. spinigerum* egg (S) with the same magnification. (From Miyazaki I., 1991).

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3.5.3 *Gnathostoma nipponicum*

The parasitic numbers of the adults usually have one, rarely two or three tumor-like mass, live in the esophagus of wheasels which is almost always located 2-3 cm apart from the stomach, and the size of the mass depends on the number of parasites in the tumor (Miyazaki, 1991). The head-bulb of adult worms is armed with 7-8 rows of cephalic hooklets, and only the anterior half of the body is covered with cuticular spines, being quite similar to *G. spinigerum* (Figure 3.25). Females measure 17-26 x 2.6 mm while males are 10-15 x 1.9 mm. However, the two species are distinct from each other in the following features of the spines (Miyazaki, 1991):

- First, the female of this species has no spines at all at the tail end.
- Second, in this species both lateral margins of the spines are roundish.
- Third, the trident-type spines (B), found in the largest number, are apparently different in shape from those of *G. spinigerum* and *G. doloresi*.

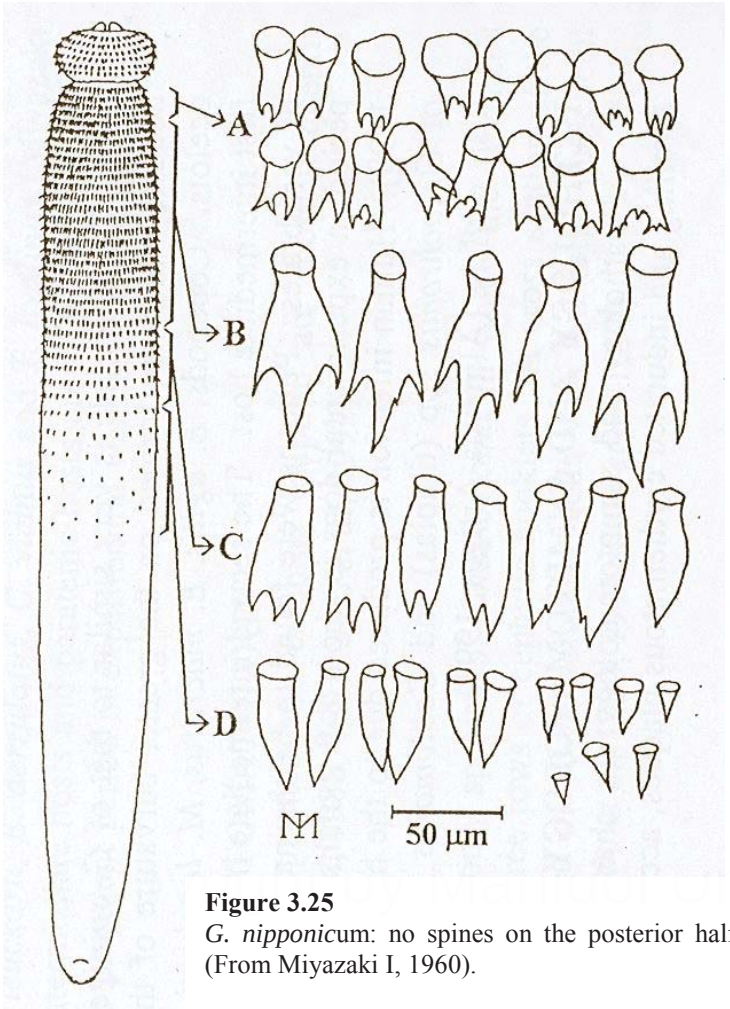

Figure 3.25
*G. nipponic*um: no spines on the posterior half of the body. (From Miyazaki I, 1960).

The spines of the male are same as that of the female, but the caudal ala is equipped with small spines, having eight pairs of large and small papillae. The morphology of the first-stage larvae is also similar to that of *G. spinigerum* but a little larger. The important feature of the early and advanced third-stage larvae is that they have only three transverse rows of hooklets on their head-bulbs (Figure 3.26). These cephalic hooklets are oblong in shape, and the average number from rows one to three is 33.4, 36.1 and 40.0, respectively (Miyazaki, 1991). The larvae measure 0.61-2.35 x 0.11-0.17 mm (Sato et al., 1992; Sohn et al., 1993). Fertilized eggs are 69-76 x 39-45 μm with a plug at one pole, are nearly the same as those of *G. spinigerum* in shape and color as well as in the dents on the eggshell, but somewhat bigger. Therefore, a fair amount of difference can be noted from the eggs of *G. doloresi*. When female worms alone are parasitic, one would naturally find unfertilized eggs (Figure 3.27), which may be readily mixed up with things other than the egg (Miyazaki, 1991).

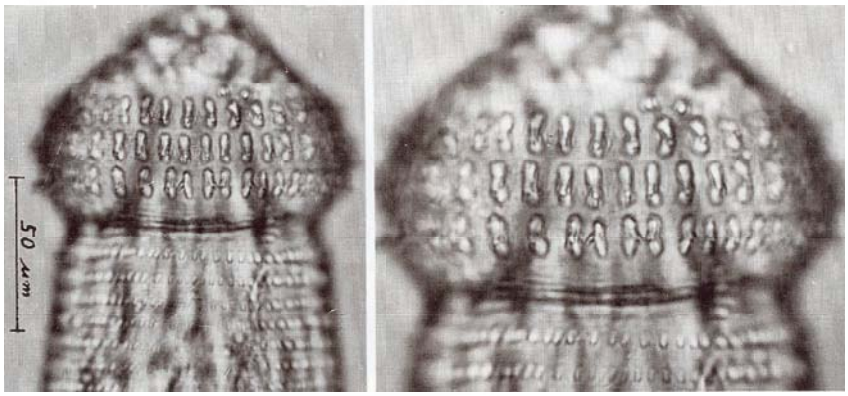

Figure 3.26 *G. nipponic*um: Anterior end of an advanced third-stage larva removed from the muscle of frog. Right: Enlarged headbulb; number of hooks per row are 24, 26 and 32 posteriorly, noted three rows of hooklet. (From Miyazaki I., 1991).

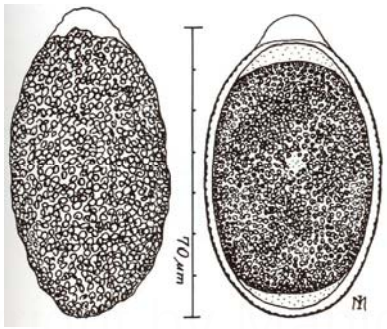

Figure 3.27 Fertilized (right) and unfertilized (left) eggs of *G. nipponic*um. (From Miyazaki I., 1991).

3.5.4 *G. binucleatum*

The general morphology of adult worms, eggs and larvae of this species most closely resembles *G. spinigerum*. The head-bulb of the adult worm is armed with 8-10 rows of cephalic hooklets. The entire body is covered with cuticular spines of different size and shape. The spines on the posterior half of the body are greatly reduced in size and distributed in a very irregular manner. Females measure 15-22 x 0.9-1.2 mm while males are 21-25 x 1.1-1.4 mm. The left spicule is 1.40 mm in length and the right one is 0.31 mm. Uterine fertilized eggs are oval, colorless, 58-68 x 37-39 μm with a plug at one pole, and the surface of their shell exhibits a fine granulation. This contrasts with the study of Koga et al. (1998) using scanning electron microscope that reported that no pits were found on the surface of the eggs. The average size of the first-stage larvae measures 194-256 μm in length. Advanced third-stage larvae measure 2.6-5.9 mm in length, bear four rows of cephalic hooklets of which the average number from rows one to four are 38.7, 42.4, 44.7 and 48.2, respectively (Almeyda-Artigas, 1991).

3.6 Life cycle

The general life cycle is identical among all the various *Gnathostoma* spp., with only slight variations occurring in the second, paratenic, and definitive hosts (Daengsvang, 1980; Chai et al., 2003). Dogs, various felines, and wild mammals serve as the definitive hosts in the life cycle of the nematode (Miyazaki, 1991). The first intermediate hosts are copepods. The second intermediate hosts are fresh-water fishes, reptiles and amphibians harboring the infective third-stage larvae. The adult parasites are found in tumor-like masses in the gastric or esophageal wall of definitive hosts that have consumed raw fish. The habitats of adult parasites differ among species; esophagus (*G. nipponicum*), stomach (*G. spinigerum*, *G. hispidum* and *G. binucleatum*) or kidney (*G. miyazakii*) of the definitive hosts of several wild and domestic mammals. Eggs are passed fecally and hatch in water to release first-stage larvae. These larvae develop into second stage larvae after being ingested by small copepods (*Cyclops* spp.), which serve as the first intermediate hosts. The second intermediate hosts include freshwater fish, eels, frogs, snakes, birds, and some

mammals that become infected by swallowing the infected copepod or other intermediate hosts. In Thailand, 48 species of vertebrates have been reported to serve naturally as the second intermediate (and/or paratenic) hosts of *G. spinigerum* (Rojekittikhun, 2005). Third-stage larvae develop and encyst in their flesh. They are passed to a wide spectrum of paratenic hosts from that point. The life cycle is complete when a definitive host ingests a second intermediate/paratenic host infected with mature third-stage larvae. Humans are accidental or paratenic hosts; they acquire infection by eating third-stage larvae encapsulated in raw or partially cooked freshwater fish, especially *Monopterus alba* (swamp eel), *Fluta alba* (eel), *Charias batrachus* (catfish), and *Channa striatus* (snake-headed fish). After being ingested, the larvae migrate through various tissues and cause various symptoms of "larva migrans." An alternative suggestion is that humans could become second intermediate hosts by ingesting infected copepods or that third-stage larvae from infected meat could penetrate the skin of food handlers, eliminating the requirement of being ingested. Nonetheless, humans represent a dead-end host because the gnathostome worm fails to mature to the adult stage. The schema of *Gnathostoma* life cycle is demonstrated in Figure 3.28.

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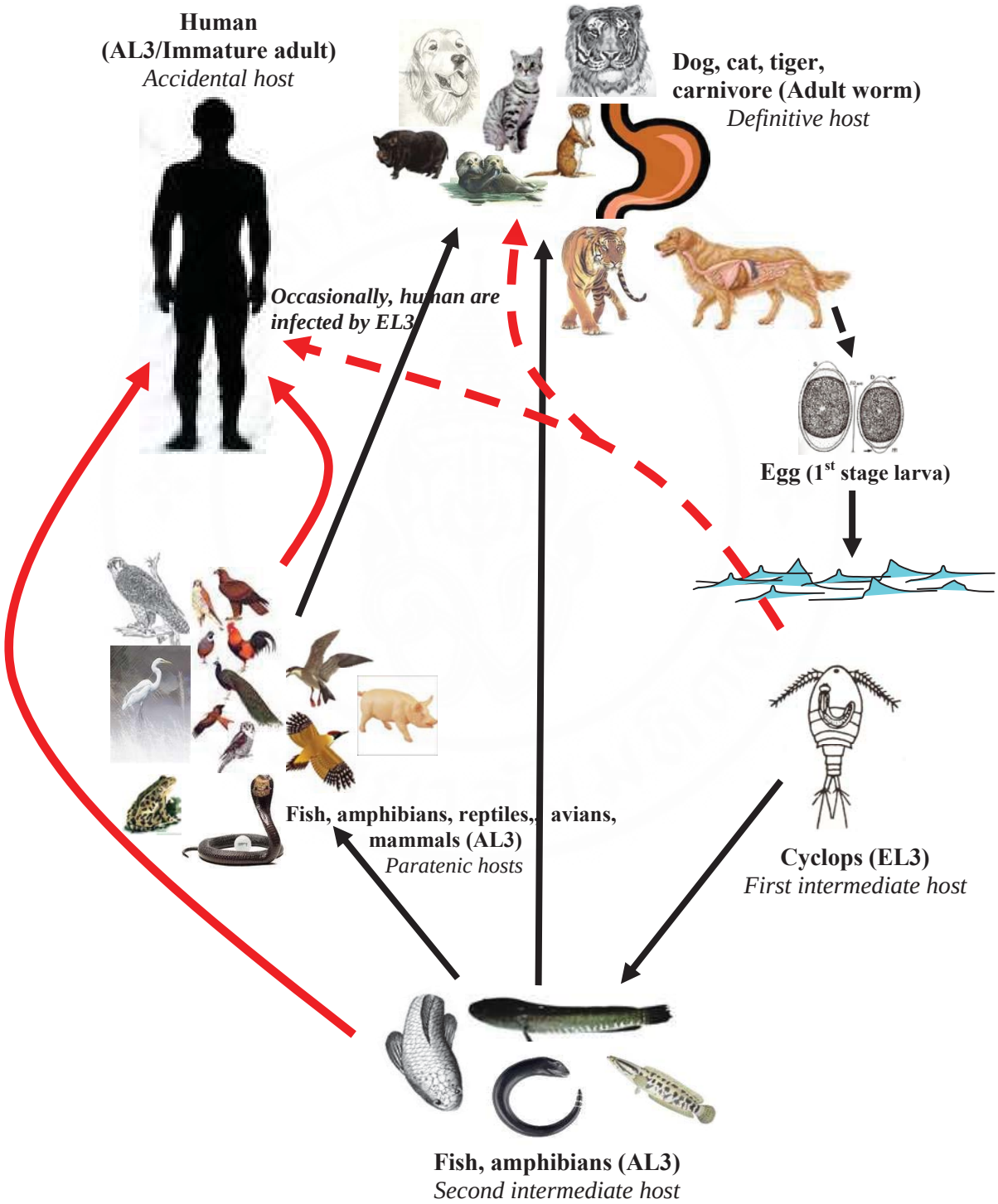

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Figure 3.28 Life cycle of *Gnathostoma* species

3.6.1 *Gnathostoma spinigerum*

Adult male and female worms inhabit in the gastric wall where they made a tumor of their hosts. Various animals of the feline and canine families can be the final host, the former being particularly important. The common definitive hosts of *G. spinigerum* are domestic cats and dogs and some other carnivorous animals (e.g., tigers and leopards) (Daengsvang, 1980; Miyazaki, 1991). In Japan cats and dogs are known to serve as definitive hosts: it was found in autopsies done in Kyushu that 27.5% of 51 cats and 4% of 643 dogs were infected (Miyazaki, 1991). These figures seem to indicate that cats are apparently more suitable as a final host. In Japanese zoos, feces were collected from tigers and leopards imported from Thailand and Malaysia, 2 out of 8 tigers and 5 out of 12 leopards were infected (Miyazaki, 1991). Adult worms usually inhabit in the host gastric wall in a group, producing a large tumor. The preferred location is near the cardia. Rarely, there are two tumors. In either case, each tumor has a small pore, through which eggs were laid inside the tumor come out to the lumen of the stomach. Highest number of adult worms living in a tumor is 22 recorded in a cat. Perforation of the gastric wall caused by the worm has been reported. The worm can rarely mature to become an adult in humans, but no report of the parasitism, suggesting that humans may not be the definitive host (Miyazaki, 1991). But recently, the first case of an adult *Gnathostoma* found in the stomach of human was reported in Nong Khai hospital, northeastern Thailand. The worm was removed from the stomach of a 41-years-old Thai woman during gastroscopy, identifying as a male *G. spinigerum* (Lertanekawattana et al., 2004). This is the first time that morphologically mature adult *Gnathostoma* was recovered from the gastric wall of a human, which leads to the question of whether humans can be a definitive host of this nematode. Their eggs passed in stool, cleave in the water, and around 27°C second-stage larvae with sheath emerge therein in a week. The larvae have molted once without taking the exuviae. The larvae swim actively in the water, and then are ingested by a copepod. They take off the sheath in the digestive tract of the copepod and migrate to the hemocoel to become complete second-stage larvae. And after the second molt they become early third-stage larvae (EL3), which take 7 to 10 days at 27°C. When a tadpole or fish eats the copepod, the early third-stage larvae leave the intestinal tracts of the new host, enter the muscle, and grow in a month or so

to be advanced third-stage larvae (AL3). While the host grows to a frog or an adult fish, the larvae remain as they are. Most larvae are coiled around their own heads, and enveloped by thin cysts of connective tissue made by the host. Some of them come out of those cysts, migrate to somewhere else, and become re-enveloped there. Such infection with an early third-stage larva in a first intermediate host (copepod) is called the *primary infection*. The animals thus infected are called the second intermediate host. On the other hand, when second intermediate hosts harboring advanced third-stage larvae in the muscle are eaten by animals other than final hosts, the larvae excyst, go through the intestinal wall of the new host, and enter the muscle again to be encysted. Such infection with advanced third-stage larvae is called the *secondary infection*. In nature, such mode of infection occurs frequently. The host in this case is called the paratenic host. The carnivorous fish such as snake-headed fish plays dual roles of the second intermediate and the paratenic hosts. Since the advanced third-stage larvae are strongly infective, they are able to change paratenic hosts one after another by successive secondary infection, e.g., from a cold-blooded animal to a warm-blooded animal, or *vice versa*. In this way, as mentioned above, the natural hosts cover an extremely wide range of animals. The advanced third-stage larvae do not change their form throughout frequent transfers between different paratenic hosts. However, when they invade a worm-blooded animal, the body is somewhat enlarged, and the reddish tone of haemolymph and the brown shade of the intestine become darker. This is because oxidized hemoglobin in haemolymph and brown granules in the intestinal epithelium increase in amount (Daengsvang, 1980; Miyazaki, 1991).

3.6.2 *Gnathostoma hispidum*

Adult worms, both male and female are found in the gastric wall of final hosts, which are domestic and wild pigs. The susceptibility differs with breeds of swine. Various copepods serve as the first intermediate host; *Eucyclops serrulatus* and *Cyclops vicinus* have been identified in Japan. The second intermediate and paratenic hosts are fish, amphibians, birds, and mammals except swine; reptiles can probably be the host. The life cycle of *G. hispidum* resembles to that of *G. spinigerum*, but it is worth noticing that the larvae of this species can hardly grow to the advanced third-stage in fish. Newly passed fertilized eggs are mostly in the 2-4 cell stage. After

excretion from pigs, eggs start growing in the water and become embryonated in 3-10 days. The larvae molt once within the egg, but without taking exuviae off to become sheathed larvae and appear in the water. When being eaten by copepods, they continue to grow and upon completion of the second molt they become early third-stage larvae. Then, after being eaten by a fish, they leave the intestinal lumen to enter the viscera and become enveloped in a cyst of connective tissue. This means that different from the preceding species, they cannot develop into the advanced third-stage larvae in fish. However, it may sometimes reach that stage in frogs after a long time. When mammals that serves as a paratenic host to ingest a fish, the larva finally grows to the advanced third-stage larva, which is coiled in a cyst in the muscle. In conclusion, larvae of *G. hispidum* remain mostly in the early third-stage within the cold-blooded animals, and become advanced third-stage larvae after entering the worm-blooded animals. The final host is infected by taking in one of the first and second intermediate and paratenic hosts, and the worm matures in two to six months (Miyazaki, 1991). It means that the definitive host is acquired by ingestion of animals harboring the advanced third-stage larvae or drinking water contaminated with copepods containing the early third-stage larvae. Human can probably be infected through various animals, but for the time being only the loach is clearly known to be involved (Miyazaki, 1991).

3.6.3 *Gnathostoma doloresi*

The final host or natural definitive hosts of this species are swine and wild boars (Daengsvang, 1980, Miyazaki, 1991). Adults of this worm are parasitic in the gastric wall, but there is no visible tumor formation in the gastric wall of the animals as in *G. spinigerum* infection in cats or dogs. The first intermediate hosts are cyclops. Second intermediate and paratenic hosts are generally amphibians and reptiles playing roles as the former and the latter, respectively. In Japan, two species of salamander, *Hynobius naevius* and *H. stejnegeri*, followed by frogs and newts have been established to be the intermediate hosts. The paratenic hosts are snakes including *Trimeresurus elegans, T. flavoviridis, T. okinavensis, Natrix pryeri*, and *Dinodon semicarinatus*, which the last snake is heavily infected (Miyazaki, 1991).

3.6.4 *Gnathostoma nipponicum*

The life cycle of this species is almost the same as of the other species. The final host is a weasel. Adult worms inhabit the esophageal wall, gathering in a spot to make a solid mass as seen from the outside. Experimentally, ferrets, belonging to the same family, can also be the final host. The first intermediate hosts are copepods such as *Mesocyclops leuckarti, Eucyclops serrulatus, Cyclops vicinus,* and *Thermocyclops hyalinus*. The second intermediate and paratenic hosts are loaches and snakes in natural infection; frogs and rats can also be the hosts experimentally (Miyazaki, 1991). The prepatent period in experimental infection in weasels is 65-90 days (Ando et al., 1992; 1994)

3.6.5 *Gnathostoma binucleatum*

The life cycle of this species is very similar to that of *G. spinigerum*. Adult worms inhabit in nodules, which are formed on the greater curvature of the stomach of feral cats or ocelots. The first intermediate hosts are copepods, *Eucyclops agilis, E. macrurus, Mesocyclops leuckarti,* and *M. edax*. The second intermediate hosts are the member of 5 classes of vertebrates. Pelicans were found to be the natural paratenic hosts. The prepatent period in experimental infection in dogs is about 8-9 months (Akahane et al., 1994; Koga et al., 1998). Human infection is produced due to the habit of eating raw fish flesh, mostly of *Oreochromis* spp. (tilapias) and *Gobiomorus dormitor*, as a traditional Mexican dish, ceviche (Almeyda-Artigas, 1991).

3.7 Mode of transmission

There are three possible ways of transmission of *Gnathostoma* species which were proven experimentally (Daengsavang, 1980; Miyazaki, 1991).

3.7.1 Ingestion

This was shown by experimental feeding of cats with infected flesh of fresh-water fish of the species: *Ophicephalus striatus* (snake-headed fish or mudfish) and *Clarias batrachus* (cat-fish) (Prommas et al., 1937; Daengsvang, 1980). The

species *O. striatus* is considered to be the most significant intermediate host causing human gnathostomiasis in Thailand and other Asian countries.

3.7.2 Skin penetration

Advanced third-stage larvae were used in experiments done on intact skin of dogs and cats. All experimental animals were infected via skin penetration. The development into adult worms was also somewhat faster by skin penetration in a few cases than by the oral route (Daengsvang et al., 1970a, b). Unequal rates of development of the worms were also observed during transient migration of larvae in the body of the animals. The larvae became immature adults before entry through the stomach where they fully developed to adults. This pattern is construed to be similar to the migration of larvae after skin penetration in man (Daengsvang et al., 1970a, b). Infection via skin, especially of persons involved in handling infected animal flesh, should be equally alarmed as infection via oral route. These experiments, moreover, were done only on the species, *G. spinigerum*.

3.7.3 Transplacental transmission

Experiments done on 22 experimentally infected pregnant white mice revealed that out of 152 offspring produced, two had one unencysted *G. spinigerum* third-stage larvae each. Reports of three cases of human gnathostomiasis in babies further suggested that gnathostome infection by *G. spinigerum* may be transmitted to fetus (Daengsvang, 1968; 1980).

3.8 Pathology and symptomatology

3.8.1 *Gnathostoma spinigerum*

The latent period, i.e., the time between the ingestion of an advanced third-stage larva and the emergence of the specific dermal symptom (migrating intermittent edema), varies from a few weeks to a year or two, with the mode being three to four weeks. Anyhow, the ingested larva penetrates the gastric wall or occasionally the duodenal wall to come into the liver. After moving around in the liver for a while, it migrates to muscles or subcutaneous regions. This makes the time of emergence of the

skin symptom quite variable. The livers seen to be important for development of the larva, as suggested by the observation that most of larvae experimentally introduced under the skin come to the liver (Daengsvang, 1980; Miyazaki 1960, 1991). Moving around in the liver, it continues to grow, causing various lesions such as destruction of the liver parenchyma, hemorrhage, granulation, and cicatrisation. This leaves trails of larva's passage, which can be seen from the surface of the liver as numerous lines, long or short and red or white. As a result, it causes hepatic hypofunctions, for which not only the mechanical destruction but also damage caused by the secretes of the esophageal gland or metabolites of the worm on by allergic reactions. In the case of humans, prodromes such as anorexia, vomiting, abdominal pain, and fever may be experienced prior to the appearance of skin lesion, which is considered as the results of the hepatic disorder.

Behavior of the worm after leaving the liver is indefinite. It moves around wherever it wants. In the case of final host, cats or dogs, it returns to gastric wall as an adult. In the case of paratenic host, chickens, rats, swine, etc., it usually become encysted in the muscle or subcutis as a larva. Humans seem to be positioned just in between, in the sense that most of the worms are in the adult form despite their intramuscular or subcutaneous parasitization. For example, out of 18 worms collected from patients in Japan, 13 including nine males were found to be in the adult form (Miyazaki, 1991). It must be stressed, however, that even after growing up to adults, none of them have been found to be parasitic in the human gastric wall, nor have been any fertilized eggs observed in human stool.

Many of the worms coming out of the human liver then move to the body surface and cause cutaneous gnathostomiasis, recognized as localized swelling that appears intermittently and moved around. The initial location is generally in the trunk, especially the abdomen. Such tumors vary in size, but usually smaller than a first or a palm. The swelling a reddish tone with fever. The patient frequently complains of itching. Pain is mild, but occasionally it is so strong as to be misdiagnosed as other diseases. Swelling lasts for various lengths of time, ranging from two days to about a week, until it subsides, but after a while it may appear in another spot. The time and

location of reappearance depend on the worm. When it approaches close to the body surface, creeping eruption occurs, from the top of which the worm occasionally can be excised. Marked inflammation is observed in the tissues of affected skin. Allergic changes are also seen, as evidenced by marked infiltration of eosinophils, which is most conspicuous when the worm is around the interface between the reticular layer of the corium and subcutaneous adipose tissues. In contrast, when the worm is proceeding through the outer layer of the corium, the infiltration is not serious.

As long as the worm migrates near the body surface, no serious disorder generally occurs, but when it enters deep and causes *visceral gnathostomiasis*, it becomes dangerous. For example, its laryngeal invasion may cause dyspnea due to edema, and orbital invasion will result in diminished visual acuity, cellulitis, exophthalmos, etc., with the worst outcome being direct involvement of the eyeball itself, which necessitates surgical removal of worms. Most dangerous form is the invasion into the central nervous system. As the worm moves around freely, the destruction of the brain tissues and hemorrhage occur to result in poor prognosis. To sum up, when the worm is in somewhere above the neck, it means high risk. Below the neck, it might cause troubles such as pneumothorax and hemoptysis in the lung, hematuria in the kidney, and granuloma formation in the peritoneal cavity, which may occasionally be misdiagnosed as a tumor. The life span of the worm in the human ranges from several to 10 years or longer; therefore, a long asymptomatic interval may be followed by a sudden recurrence of manifestations (Daengsvang, 1980; Miyazaki, 1991).

3.8.3 *Gnathostoma hispidum*

The pathology and symptoms of this species in a human is nearly the same as those of gnathostomiasis spinigera, but creeping eruption tends to be observed more frequently in infection with this species. This is because the worm migrates close to the body surface. However, it is not yet cl ear whether such a tendency specifically associated with the infection with small early third-stage larvae or whether it is seen in possible infection with large advanced third-stage larvae as well. Also in this disease, the patient complains of abdominal symptoms frequently at an early stage, caused by

invasion of larvae into the liver, and leukocytosis with marked eosinophilia can be seen in peripheral blood. While repeated recurrences over a long period of time are not infrequent in gnathostomiasis spinigera, such a tendency is rather weak in this disease probably because the life span of the worm in the human body is short. This short life span might possibly be related to the infection at the stage of premature early third-stage larva. Anyhow, as gnathostomiasis hispida has only recently been found, its detailed characteristics await further studies (Miyazaki, 1991).

3.8.3 *Gnathostoma doloresi*

Creeping eruption and/or migratory localized swelling with redness, called 'Quincke's edema' are most common features of *G. doloresi* infection (Table 3.3) Epigastric pain, chest pain, slight itching and high fever are also noted in some patients. Leukocyte counts are normal or slightly elevated with moderate to high eosinophilia (9-67%) (Ogata et al., 1988; Nawa et al., 1989, 1997; Akahane, 1998).

3.8.4 *Gnathostoma nipponicum*

Clinical symptom with itching, erythematous patches and creeping eruption are most frequently observed in patients of this disease. (Ando et al., 1988, 1991; Sato et al., 1992).

3.8.5 *Gnathostoma binucleatum*

Pathology and symptomatology are characterized by intermittent migratory swelling and indurated erythematous plaques, accompanied by itching and occasional pain. Creeping eruptions were also noted in about one-third of patients. In most cases, relapse was observed over several years. Ocular involvement was also reported in two cases (Camacho et al., 1998; Ogata et al., 1998; Vargas-Ocampo et al., 1998; Ruiz-Maldonado & Mosqueda-Cabrera, 1999).

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Table 3.3 Clinical features of human gnathostomiasis caused by different species

Species	Duration	Affected site	Skin lesion
G. spinigerum *G. binucleatum*	> 1-4 years	Peripheral extremitetes, face, head	Erythema > Creeping
G. hispidum *G. nipponicum* *G. doloresi*	< 2-3 months	Central abdomen, back	Creeping > Erythema

(Modified from Table 2, Nawa, 2002)

3.9 Diagnosis approaches

Definitive diagnosis of human gnathostomiasis requires discovery of the larvae of the nematodes from the lesions (Chai et al., 2003; Nuchprayoon et al., 2003). Morphological study is important for the identification of *Gnathostoma* species. Biopsy of a skin lesion can yield sufficient information to make diagnosis and is curative, but biopsies frequently miss the larvae in migrating lesions (Miyazaki, 1991). Probable cases are diagnosed by a history of eating raw or partially cooked fish or other second and/or paratenic hosts. The clinical manifestation of external and visceral gnathostomiasis such as subcutaneous or cutaneous intermittent migratory swelling, and blood eosinophilia can be striking, with more than 50% of circulating leukocytes (Daengsvang, 1980; Rusnak et al., 1993). Immunodiagnosis is useful to confirm for human gnathostomiasis (Dharmkrong-at et al., 1986; Mimori et al., 1987; Anantaphruti, 1989; Chaicumpa, 1997; Camacho et al., 1998; Anantaphruti, 2002; Dekumyoy et al., 2002; Laummaunwai et al., 2007).

3.10 Immunodiagnosis of human gnathostomiasis

As noted that, the definitive diagnosis for human gnathostomiasis is based on the presence of worms from the affected sites, but the frequency of successful

biopsy was estimated to be less than 50% (Miyazaki, 1991; Nawa, 2002). Several immunodiagnostic tests have been developed for the diagnosis of gnathostomiasis over several decades (Chaicumpa, 1997; Anantaphruti, 2002a; 2002b). The conventional method was the skin test, which used in the period of the 1960s (Daengsvang, 1980). The volume of 0.05cc of a 50,000-fold saline solution of antigen prepared from the larvae of *G. spinigerum* or *G. doloresi* is injected intracutaneously with the 100% of sensitivity, but their Ag. cross-react with Ab. Paragonimus and Schistosome (Daengsvang, 1980; Anantaphruti, 2002b). The precipitation test, gel diffusion, radioimmunoassay, complement fixation test, indirect hemagglutination antibody test (IHA) and indirect flurescent antibody test (IFA) were also developed by many investigators. However, those methods are not commonly use in practical works (Anantaphruti, 2002b). Enzyme-linked immunosorbent assay (ELISA) to detect total immunoglobulin G (total IgG) has been widely used in the endemic countries. Crude somatic extracts of either larvae of *G. spinigerum* or adult worms of *G. doloresi* are used as the detecting antigens (Dharmkrong-at et al., 1986; Mimori et al., 1987; Anantaphruti, 1989; Camacho et al., 1998). Nopparatana and colleagues purified the antigen prepared from *G. spinigerum* advance third stage larvae (GsAL3) by gel filtration, chromatofocusing and anion exchange chromatography; the enriched 24 kDa molecular weight antigen showed 100% sensitivity and specificity in five parasitologically diagnosed gnathostomiasis patients. Moreover, the Comparative studies of crude and monoclonal antibody affinity-purified by ELISA have been revealed that no difference was observed with sensitivity, specificity, and positive and negative predictive values were 100%, 98.4%, 87.5% and 100%, respectively (Chaicumpa et al., 1991; Rojekittikhum et al., 1991; 1995). Whereas, human gnathostomiasis has been diagnosed in an excellent evaluation of immunoblot to detection of total IgG and continued finding sensitivity and specificity were 91.6% and 87.8%, respectively. Although IgG4 had higher specificity, it is too costly for routine immunodiagnosis (Anantaphruti, 2002a; Dekumyoy et al., 2002; Anantaphruti et al., 2005; Laummaunwai et al., 2007). The cross-reactivity to other parasitic antibodies was tested, and the 24 kDa of total IgG elicited from crude somatic extract of GsAL3 is still the specific diagnostic band for human gnathostomiasis (Nopparatana et al., 1991; Tapchaisri et al., 1991; Dekumyoy et al., 2002).

3.11 Treatment

No efficacious drug, non appropriate protocol is presently available. Surgical removal of the worm is the only practical treatment for gnathostomiasis when the surgery can be safely done (Daengsang, 1980; 1986). Combinations of supportive, symptomatic, and anti-inflammatory treatments are preferable. Anyhow, albendazole 400mg has been used to treat gnathostomiasis and found to have minimal side effects, twice daily or once daily for 21 days, giving a cure rate of 93.9% and 94.1%, respectively (Kraivichian et al., 1992). The worm tended to migrate outward to the skin of the human host after chemotherapy, and then was easily removed by picking out with a needle or just a scratch with the patient's nail (Suntharasamai et al., 1992).

3.12 Prevention

The most important preventive measure is the avoidance of eating fresh meat from second intermediate or paratenic hosts, particularly raw or undercooked freshwater fish from endemic areas. The larvae appear to be killed by freezing infected meat to –20°C for 3 to 5 days. Vinegar appears to kill the *Gnathostoma* larvae in approximately 6 hours and soy sauce in 12 hours, but limejuice is not effective after 5 days at room temperature or after 30 days at 4°C (Daengsvang, 1980; Rusnak et al., 1993).

3.13 Other *Gnathostoma* species

3.13.1 *Gnathostoma turgidum*

Stossich (1902) first described this parasite from the opossum, *Didelphis azarae* (= *D. paraguayensis*) in Argentine, and 20 odd years later Travassos (1925) found this species in Brazil from another species of opossum, *D. aurita*. Moreover, *G. turgidum* was found in the USA from another opossum, *D. virginiana*, by Dickmans (1931) in Louisiana, followed by Chandler (1932) in Texas. Chandler thought it to be distinct from the South American species and named it *G. didelphis*. However, upon detailed examinations of the type specimen (male) by Miyazaki (1960), both turned

out to be of the same species. This *Gnathostoma* has also been obtained from *Philander opossum* in Panama, while Miyazaki et al. (1978) found it from *D. marsupialis* in Peru. Thus, this *Gnathostoma* is considered to be widely distributed in the American Continent, with various opossums as the final host (Miyazaki, 1991; Camacho et al., 2008; Nawa et al., 2009).

Adults are parasitic in the stomach of the opossums. They make tunnels on the thickened gastric walls, showing a part of the body in the gastric cavity. It is rather hard to pick out the worm without damage. On the body surface, cuticular spines are noted in the anterior half, of which the typical ones have several teeth on the free margins. The egg is the same as other gnathostome species in color and the dents on the eggshell, but rather characteristically both poles have bulges (Miyazaki, 1991).

3.13.2 *Gnathostoma procyonis*

It was described by Chandler (1942) from the raccoon, *Procyon lotor*, in Taxas, USA, and later found from the same animal in Georgia, Louisiana and Florida. In particular, raccoon in some marshland of South Louisiana are densely infected. Occasionally, infection is also noted in the skunk, *Mephitis mephitis* (Miyazaki, 1991).

The state of parasitism is similar to those of *G. hispidum* and *G. doloresi*, with the anterior part of the body inserted into a tumor on the gastric wall. The adult is covered with cuticular spines all over the body. *G. procyonis* is divided into a smaller class in the posterior half of the body and a bigger class in the rest. This make easy to differentiation from the two species with spines on the whole body surface, *G. hispidum* and *G. doloresi*. Furthermore, the morphology of the trident spines is also of diagnostic value. At the tail end of the male, there are eight pairs of large and small papillae, and the right copulatory spicule is about 1/3 of the length of the left one. The average size of egg is 71 x 39 μm, with a bulge at one pole only like the eggs of most *Gnathostoma* spp. (Miyazaki, 1991).

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Three species of copepods have been identified as the first intermediate host. The second intermediate hosts or paratenic hosts in nature are three snakes, four

turtles, and one crocodile, *Alligator mississippiensis*, and one fish, *Amia calva*. The course of development is the same as those of the other species. The advanced third-stage larvae are encysted mainly in the muscle of the above-mentioned reptiles and fish. The headbulb hooklets are similar in shape to those of *G. spinigerum*, are also aligned in four rows, but their numbers in these rows are less than those for that species, averaging 32.7, 36.6, 41 and 45 from front to rear (Miyazaki, 1991).

3.13.3 *Gnathostoma miyazakii*

This species was discovered by Anderson (1964) as a new species from the kidney of the otter, in Ontario, Canada. Later it was also found from the same mammal in North Carolina, USA. Both the male and female are about 4 cm long, the anterior half of the body being covered with cuticular spines with several teeth, which is similar to *G. turgidum*. The posterior half of the body shows circular folds, but on the ventral side of the male caudal ala tiny processes are arranged in lines. That specific in this species is the tail end. Both in the male and female, the part beyond the anus or cloaca is stretched long and narrow. The egg is 69 x 42 μm on the average, with a bulge at one end only like those of most *Gnathostoma* spp. (Miyazaki, 1991).

3.13.4 *Gnathostoma malaysiae*

It was described from two species of rats, *Rattus surifer* and *R. rattus tiomanicus*, caught at a highland of 3.000 ft above the sea level in the Tioman Island off the shore of Pahang, Malaysia, and later found also from *R. surifer* in Thailand. Each adult makes an individual diverticulum on the wall of the rat stomach and inserts the anterior part of the body into it, holding the posterior part free in the gastric lumen, which portion is apt to swell. The entire body is covered with cuticular spines mostly with three or four teeth, which are sharply divided into a smaller class in the posterior 1/3 of the body and a bigger class in the rest. The part with the smaller spines corresponds to that which is apt to swell. At the tail of a male worm, small spines grow densely, leaving little place without a spine. It is fairly characteristic that of the pedunculate papillae the first and second ones are smaller than the thirds one. The egg measures 67.5x36.0 μm on the average, of both ends of which have bulges. The color and surface structure are the same as those of others.

3.13.5 *Gnathostoma vietnamicum*

It was first described by Le-Van-Hoa (1965) from the kidney of the otter, *Lutra elioti*, in Viet Nam, and later found by Daengsvang (1973) from another otter, *Aonyx cinerea*, in Thailand. This worm, like *G. miyazakii*, possesses a strange habit of being parasitic in the kidney of otters, in which it inserts its anterior part into the renal parenchyma, with the posterior part being free in the renal pelvis. In Thailand, otters have been reported in which the worms are parasitic in the ureter or urinary bladder. One of the features of this worm is unusually numerous head-bulb hooks generally aligned in 14-16 rows. The anterior one-third of the body is covered with cuticular spines similar to those of *G. turgidum*, but the rest has virtually no spine and is apt to swell. As in other species, the tail of a male has densely grown small spines on its ventral side. The egg is rather large, measuring 77.8 x 42.7 μm on the average, and has bulge at one end. The surface structure of the egg is the same as others. As the first intermediate host, *Mesocyclops leuckarti* has been identified. The head-bulb hooks of the early third-stage larva are aligned in four rows. At least some fish serve as second intermediate hosts.

3.13.6 *Gnathostoma americanum*

This was found from the stomach of *Felis tigrina* in Brazil. Although it resembles *G. spinigerum* in that felines are the host and that the form of the cuticular spines are the same, it can be easily differentiated by the presence of bulges on both ends of the egg.

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3.14 *Gnathostoma* species confirmation of the advanced third-stage larvae

3.14.1 Key for species identification on morphological of advanced third-stage larvae

The morphological identification of the advanced third-stage larvae of *Gnathostoma* species was used for second intermediate hosts study. Their characteristic features had been followed to the description of Waikagul and Thairungroj (1997).

1. 3 rows of cephalic hooklets on headbulb……... *G. nipponicum*
   4 rows of cephalic hooklets on headbulb……....(2)
2. Number of hooklets in each row less than 40,
   roundish / irregular square based hooklets,
   size increases posteriorly…… *G. doloresi*
   Number of hooklets in each row less than or more than 40……. (3)

3. Oblong cephalic hooklets…….. (4)
   Rectangular cephalic hooklets,
   size 8 x 6 μm (except 1st row,)
   oblique pyramid-like base……..… *G. hispidum*

4. Singly pointed body spines……..…… *G. spinigerum*
   T-shaped body spines……..... *G. vietnamicum*

3.14.2 Histological section of the intestinal larvae

The number and the shape of intestinal cells, and the number of nuclei in cells and also the dimension of nuclei are considered for *Gnathostoma* species identification.

G. spinigerum, intestinal epithelial cells are cylindrical in shape, each of the cells has plural number of nuclei, about 2-6 nuclei and numerous brown granules, which gives the whole intestine a brown color. Between the striking contrast of the colorless large lateral lines and the surrounding haemolymph with reddish color is

noted to be important in making diagnosis with histological sections. *G. hispidum,* the structure of intestinal epithelial cells is spherical and have single nucleus. *G. doloresi*, these species are similar in form to those of *G. hispidum*, but generally have two nuclei, ranging 0-3. *G. nipponicum*, the cells are columnar which composed of 10-14 cells. Each cell has 0-5 massive nuclei, predominantly 1-2 nuclei (Akahane et al., 1986; Ando et al., 1990; Miyazaki, 1991). *G. binucleatum* has 17-25 columnar epithelia. Each cell composes of 0-7 nuclei, predominantly 2 nuclei (Almeyda-Artigas, 1991).

3.15 Immune response in human gnathostomiasis

The host immune response to a parasite may be divided into two major categories. The first is immunoprotective, which control parasite growth and development; and minimizes parasite-induced pathology. The second aspect of the immune response produces pathology. These two categories involve multiple immune mechanisms and need not be consonant, either kinetically or mechanistically. Clinical disease is most salient when immunopathologic mechanisms are dominant or effective control of the parasite is not obtained.

As the *Gnathostoma* infection, the larvae cannot further develop into the adult form in human. The third-stage larvae can only migrate within the body of hosts; clinical symptoms of gnathostomiasis then occur because of the inflammatory reaction provoked by these migrated larvae (Miyazaki, 1960; Chaitanondh, 1978; Daengsvang, 1981). Both cell-mediated immune responses and humoral immune responses against the third-stage larvae have been demonstrated in animals and man (Anantaphruti et al., 2005). Protein components of GsAL3 extract is highly complex, comprising of more than 40 polypeptides, among which more than 20 components are antigenic in human. The relative MW of the proteins ranging from 13 to 150 kDa with the major antigenic bands at 150, 135, 120, 94, 84, 82, 72, 55, 54, 49, 43, 38, 35, 32, 28, 26, 25, 24, 21 and 20 kDa (Nopparatana et al., 1988; Nopparatana et al., 1991; Tapchaisri et al., 1991; Wongkham et al., 2000; Dantrakool et al., 2001; Anantaphruti et al., 2005). Eosinophilia is a common finding in tissue parasitic infections including

gnathostomaisis (Daengsvang, 1980). Elevated *G. spinigerum*-specific IgG and IgE antibodies have been previously shown in patients with gnathostomiasis (Suntharasamai et al., 1985; Dharmkrong-at et al., 1986, Soesatyo et al., 1987; Nuchprayoon et al., 2003; Anantaphruti et al., 2005). IgG antibodies in CSF were correlated with cerebral gnathostomiasis (Tuntipopipat et al., 1989). ELISA detection of IgG1 antibody to crude antigen extracted from GsAL3 could be reliable laboratory screening, and the diagnosis could be confirmed by the presence of IgG2 antibody to crude antigens (Nuchprayoon et al., 2003). Recently, detection of IgG antibody to protein components of crude antigen extracted from GsAL3 by immunoblot technique revealed that IgG4 in each human gnathostomiasis sera was significantly reacted with 21 kDa band. While the 20 kDa and 24 kDa protein bands were addition diagnostic bands for confirming diagnosis of infection where the 21 kDa band was fainted (Anantaphruti et al., 2005).

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CHAPTER IV
MATERIALS AND METHODS

4.1 Study site

Three provinces, Bokeo, Vientiane capital, and Champasack were selected as the representative of the northern, central and southern part of the country, respectively. Bokeo is the smallest province in the country, approximately 900 km north of Vientiane Capital and shared border with Thailand and Myanmar where is known as "The Golden Triangle". In Bokeo, Phibounthong village, Houeixay district, was selected as the study area. Phibounthong village is 4 km away from Houeixay downtown, and has 601 inhabitants (299 females). Vientiane Capital is in the center of Laos where the gnathostomiasis cases were reported (Phoumindr, 1999). Naxon village in Parkngeum District was selected as the study area in Vientian Capital. Naxon Village is 50 km from the city, and has 1,676 inhabitants (776 females). Champasack province is 700 km south of Vientiane Capital. Nongtearnoy village in Phonthong District was selected as the study area. Nongtearnoy village is 5 km from Pakse city of Champasack province, and has 977 inhabitants (510 females), but it's take more than one hour for going to the village in raining season. All three villages were selected based on the information from hospital-based study for gnathostomiasis cases. Figure 4.1 shows the location of the study areas.

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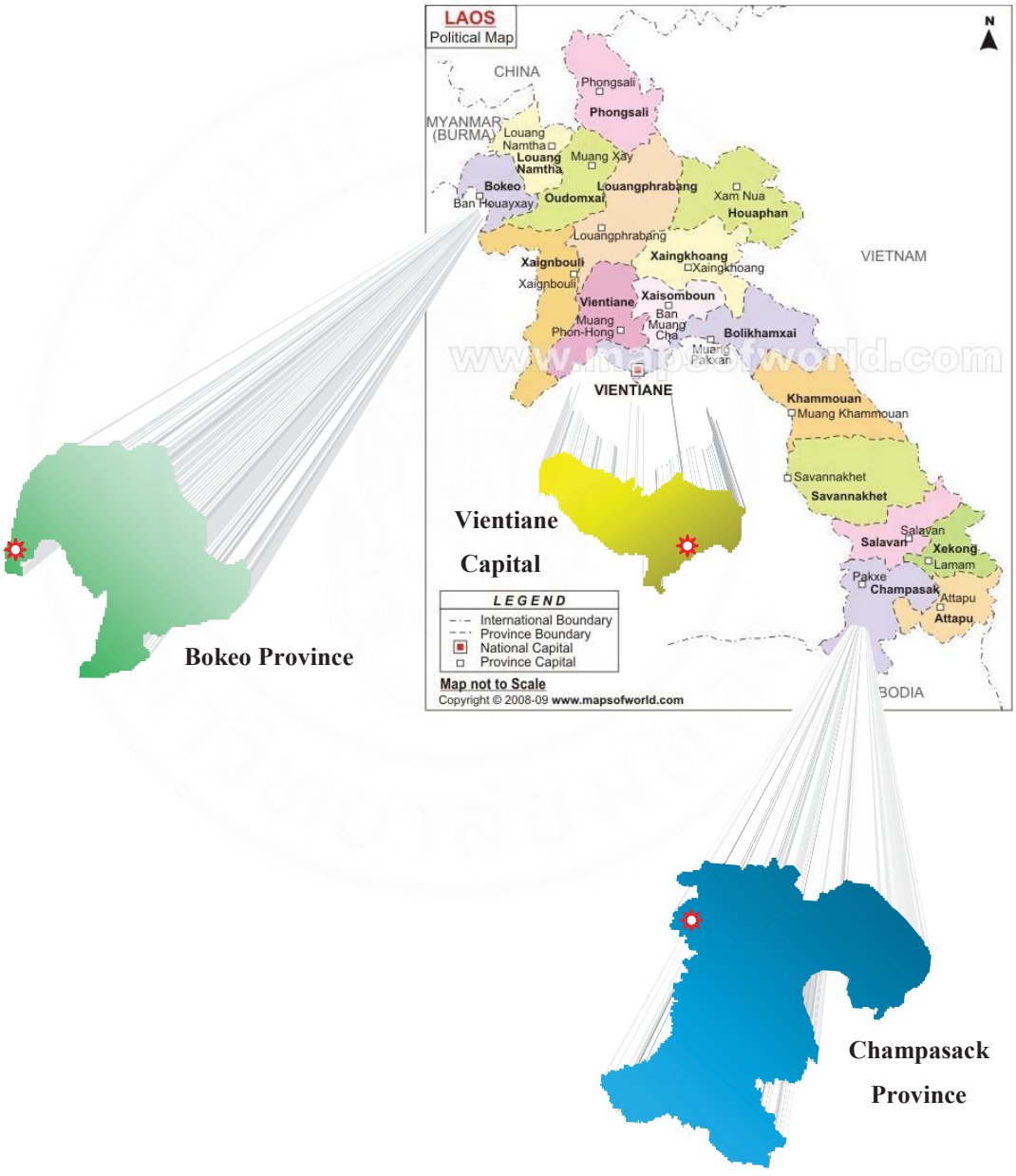

Figure 4.1 Map of Lao PDR and the study areas, Bokeo, Vientiane Capital and Champasack Province.

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4.2 Study design and population

Cross-sectional study was performed during August 2008 to September 2009. One each of the representative village was selected from three geographical parts, north, central and south, of Laos PDR primarily based on the investigation for the hospital records of gnathostomiasis/suspected cases and reviewing of the literature.

This study, hospital- and community-based studies were carried out. District, provincial hospitals and communities were selected as the study area after provincial selected.

Hospital-based study:

Hospital-based survey served to identify patients with specific clinical symptoms of gnathostomiasis which was an indicator for the location of transmission areas. Therefore, five provinces such as Bokeo, Luangprabang, Vientiane Capital, Savannakhet and Champasack province were performed the serum screening of gnathostomiasis/suspected cases in the district and provincial hospitals. This survey was conducted before community study.

In the north, no suspected cases were found in the hospital record. Therefore, as the preliminary screening, we examined 27 donated serum samples from Houeixay Hospital, Bokeo Province, and found 3 sero-positive samples for gnathostomiasis, hence this site was selected in the north.

Community-based study:

Community-based study was divided into two categories: human and animal studies as following.

- After hospital-based survey, villagers in the target communities were invited to be participants in this study. The questionnaires with closed questions for the structured interviews were developed to meet the purpose of the study.

- The second intermediate hosts (fish, swamp eel, frog) and the stools of definitive hosts (dog) were collected from the areas surround the target communities.

4.3 Sample size estimation

4.3.1 Serum samples

Sample size was determined by calculation at 50% infection with a 5% level of accuracy desired and 95% confidence level. As the study population, a total of 420 people (140people/village) were randomly selected. Before starting, the villagers were informed the objectives and process of the study. The ages of the villagers ranged from 10 to 70 years old and no pregnancy by asking, then the listed villagers was randomly selected till reached the purpose of calculated sample size. In addition, 172 voluntary participants who were not randomly selected as cohort were also examined but had not been interviewed. Sample size was identified by the following formula (Lemeshow et al., 1990).

$$\mathbf{n = Z^2\ pq/\ d^2}$$

n: the sample size

z: the standard normal deviate (1.96 for a 95% confidence level)

p: the estimate positive rate (p = 0.5= 50%)

q: the proportion of population that does not have the characteristic (q = 1-p = 0.5)

d: d = 0.05 = 5% the level of accuracy desired, or sampling error, or one-half the width of the confidence interval.

$$\mathbf{n = \frac{(1.96)^2 \times 0.5 \times 0.5}{(0.05)^2} = 384.16}$$

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Sample size was calculated as 384.16, and enrolled not less than that number. Therefore, **420 people** were included in this study, **140 people/province.**

4.3.2 Second intermediate and definitive hosts samples

The number of second intermediate and definitive hosts was given by quota and collected as much as possible.

4.4 Data collection

4.4.1 Serum sample collection and interview

Hospital-based study:

Screening questionnaires were distributed to the provincial and district hospitals of three provinces of the study. Patients, who showed the clinical symptoms of gnathostomiasis as much as available for 1 or 2 months, such as intermittent subcutaneous migratory swelling were asked for blood collection, then the blood were centrifuged or let them stand at room temperature for 2 or 3 hours to derive only serum samples and the sera were kept in -20°C or refrigerator until use. Serum samples were brought with icebox to the Immunodiagnostic Unit for Helminthic Infections, Department of Helminthology, Faculty of Tropical Medicine, Mahidol University for diagnosis of *Gnathostoma* infection using immunoblot technique. After diagnosis the patients were indicator for the local transmission areas then used to select the target villages.

Community-based study:

After selection of the target villages, all people in the target communities were got randomly sampling. Before starting, the villagers were informed the objectives and process of the study. The ages of the villagers ranged from 10 to 70 years, old and no pregnancy by asking, then the listed villagers was randomly selected till reached the purpose of calculated sample size. In addition, 172 voluntary participants who were not randomly selected as cohort were also examined but had not been interviewed.

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The randomly selected participants were interviewed by questionnaire for getting the information of risk factor affecting related to *Gnathostoma* infection and

also were collected blood samples. The collected bloods were done as the method described in hospital-based study.

4.4.2 Second intermediate and definitive hosts collection

Fish as the possible second intermediate hosts were collected from each target villages and local markets then were examined using compression technique for larvae detection.

The dropped stools of definitive hosts (dog) were collected from each target villages then examined in the field by using Kato-Katz thick smear for *Gnathostoma* egg detection.

4.5 Preparation of *G. spinigerum* antigens

4.5.1 Collection of *G. spinigerum* advanced third stage larvae

GsAL3 were harvested from the liver of freshwater eels purchased in the markets in Bangkok. Eels' livers were chopped into small pieces and then homogenized in distilled water by blender for few minutes. The homogenate was digested by 1% acid-pepsin solution (1% HCl and 1% pepsin) in a water bath with frequent stirring at 37°C for 2-3 hrs. After simple sedimentation with tap water, the larvae were collected under a dissecting microscope and washed many times with distilled water (Dekumyoy et al., 2002). Larvae were stored at -70°C until use. The process shows in Figure 4.2.

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Figure 4.2 Procedure of GsAL3 collection.
(Courtesy of Assoc. Prof. Paron Dekumyoy)

4.5.2 Preparation of crude antigens of the infective larvae

GsAL3 were ground with alumina powder in distilled water in a mortar with pestle under cool condition. The homogenized parasite suspension was sonicated with the Ultrasonic Processor XL Sonicator for 10 min at 1 min intervals for 8 min, and centrifuged at 10,000 revolutions per minute (rpm) for 60 min at 4°C (Labnet, Germany). The supernatant was collected and assayed for protein concentration by

Coomassie Plus Protein Assay Reagent Kit (Pierce, USA) and used as crude antigen extract (Dekumyoy et al., 2002). (Figure 4.3)

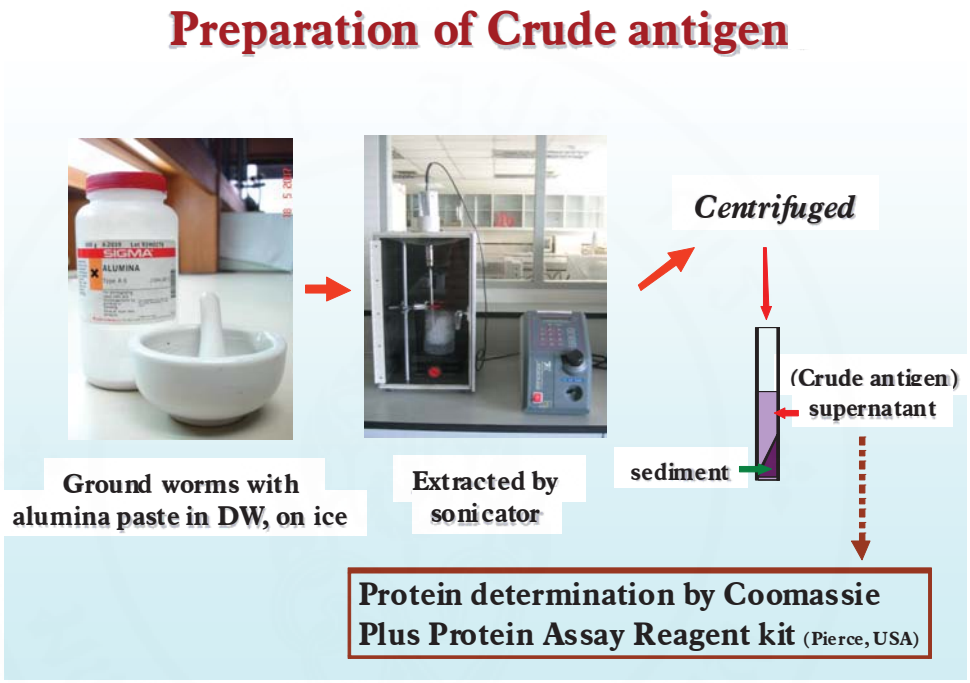

Figure 4.3 Procedure of GsAL3 crude antigen preparation.
(Courtesy of Assoc. Prof. Paron Dekumyoy)

4.6 Immunoblot technique

4.6.1 Sodium dodecyl sulphate-polyacrylamide slab gel electrophoresis (SDS-PAGE)

Proteins of the crude soluble extract of GsAL3 were separated according to their molecular weights by sodium dodecyl sulphate-polyacrylamide slab gel electrophoresis (SDS-PAGE), consisting of 5% stacking (upper gel) and 13% separating (lower gel) gels. A single percent gel was prepared in a vertical slab gel apparatus (ATTO, Tokyo, Japan). 13% separating gel was poured into the apparatus and overlaid with DW and allowed to polymerize for 1 hr at room temperature. After DW was removed, a 5% stacking gel solution was prepared and poured on the top of

separating gel, and then a single well comb was inserted into the gel. Polymerization of stacking gel was allowed for at least 1 hr. A volume of individual antigen was treated with an equal volume of sample buffer (1.5X; 0.25 M Tris-HCl, pH 6.8, 1.75% SDS, 3.75% β-mercaptoethanol, 7.5% glycerol and 0.008% bromphenol blue) then heated at 100°C for 3-5 minutes in dry bath (Accublock^{TM}, Labnet). Protein molecular weight standards of 97.4, 66.2, 45, 31, 21.5 and 14.4 kDa (Bio-Rad) were separated in parallel electrophoresis for determining the molecular weights of the antigens. A quantity of antigen was put into a single-well stacking gel. The separation of all samples was done with a constant current at 20 mA until the tracking dye reached the bottom of the gels. The protein was stained with Coomassie brilliant blue and/or silver. The molecular weights (MWs) of the stained bands were calculated by comparing their relative mobility against those of protein markers.

4.6.2 Blotting technique

The antigen was electrophoretically separated at 20 mA for a whole gel. After electrophoresis, the resolved polypeptide bands in the gel were transferred electrophoretically to a nitrocellulose membrane (0.45 µm, Protran Schleicher & Schuell BioScience GmbH, Germany) with a constant current at 450 mA for 4 hrs. Then, the nitrocellulose membrane was stained with Ponceau S solution (Sigma) and washed with distilled water to remove overstaining. The protein standards on membrane were cut and kept dried for further determination of diagnostic band (24 kDa). The blotted nitrocellulose membrane was immersed into 2% skim milk in PBS-0.02% NaN_3 for 1 hr on a rocking platform to block the non-specific binding sites on the membrane. The membrane was cut into 2 mm strips for immunoreaction. Each strip was individually reacted with 1:50 diluted sera in PBS-T-0.02% NaN_3 at room temperature overnight on a rocking platform. After washing twice with PBS-T (15 min each time) to remove the unbound antibodies, the strips were incubated with 1:1,000 diluted HPP-labeled anti-human IgG rabbit IgG (Maker Country) for 2 hrs on a rocking platform, and washed as above. The enzyme activity was allowed to develop by adding 2, 6-dichlorophenol indophenol substrate containing hydrogen peroxide for few minutes. The reaction was complete when dark brown bands appeared on the nitrocellulose strips. The reaction was stopped by washing the strips with distilled

water to remove the excess and background stain, and the strips were dried at the room temperature (Dekumyoy et al., 2002).

4.7 Molecular weight determination

The calculation of relative mobility of unknown proteins and protein standards were compared with a curve of protein standard markers from the method described by Weber and Osborn (1969), and Davies and Stark (1978). SDS-PAGE low molecular weight standards (Bio-Rad); phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and α lactalbumin (14.4 kDa), were used in this study. A linear relationship was obtained by plotting the relative mobility (Rm) of standard markers against logarithmic values of their molecular weights (Figure 4.4). The relative mobility was calculated according to the following formula:

$$Rm = \frac{\text{distance of protein band (a)}}{\text{distance of reference point (b)}}$$

a = distance between starting point (upper rim of separating gel) and the center of a band

b = distance between starting point and the ending point (dye front or a line under/over dye front)

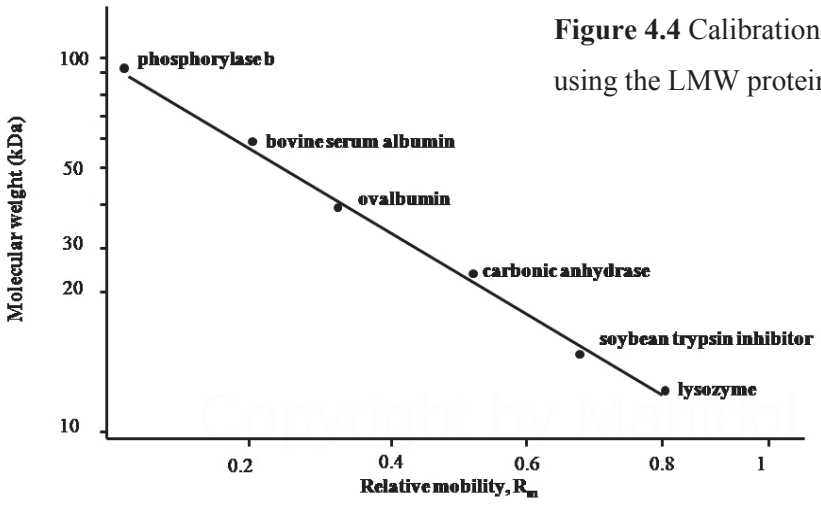

Figure 4.4 Calibration curve was constructed using the LMW protein standards.

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For a standard control, a strip of each lot of blotted sheet was treated with monoclonal antibody against 24 kDa antigen, which was detected with anti-mouse IgG-peroxidase conjugate; 1:1,000 and then development reagent was done the same as above.

4.8 Data analysis

The Data were entered in SPSS (Version 13) and analyzed in the STATA (Version 10).

Univariate analysis was used to describe the frequencies and percentages of categorical variables and mean, median and standard deviation for continuous variables.

Then, bivariate analysis was used for analyzing one dependant variable and one independent variable at a time and tests the differences and associations using Pearson's chi-square and Fisher's exact test. Fisher's exact test was used when each cell has an expected frequency smaller than 5.

Finally, the association between *Gnathostoma* infections and the risk factors were tested by multivariable analysis using logistic regression test with factors having a $P<0.05$ in bivariate analysis. Significant risk factors in multivariable analysis were expressed as an odds ratio with 95% confidence intervals (CI).

Serum samples from the participants were examined by using immunoblot technique at the Department of Helmintology, Faculty of Tropical Medicine, Mahidol University, Bangkok (Thailand).

Fish, swamp eels, and frogs were examined by compression method for GnAL3 detection. Kato-Katz thick smear were also used to detect *Gnathostoma* egg

from dog feces. Species of larvae, and egg of parasite from 2nd intermediate and definitive hosts were identified and recorded.

4.9 Treatment for the participants

All sero-positive cases of *Gnathostoma* infection were treated by albendazole 400mg a day for 21 conservative days (Nontasut et al., 2000). Single dose of albendazole 400mg was given for nematode-egg positives and 25 mg/Kg of Praziquantel for trematode-egg positives from the result of stool examination (Bunnag and Harinasuta, 1980; 1981; Albonico et al., 1994).

4.10 Ethical consideration

This study was approved by the Ethics committee in the Faculty of Tropical Medicine, Mahidol University (MUTM 2008-028-01) and the National Ethics Committee For Health Research, Ministry of Health, Lao PDR (No 184/NECHR). All procedures were explained to the participants before starting any medical activities. The consent and assent forms were presented to the participants for signing on during the interview.

4.11 Significance of the research

This study has determined the attributed fraction of tissue nematode infections of overall morbidity and predict community morbidity burden due to *Gnathostoma* spp. Moreover, the animal *Gnathostoma* infections also described. This disease is one of the main problems due to misdiagnosis with other diseases. Gnathostomiasis, so far the diagnosis is only based on clinical symptoms of intermittent cutaneous migratory swellings that are common found in almost every case. It may also cause eosinophilic meningitis syndrome which is occasionally fatal. Immunodiagnostic assay that were used in this study can differentiate *Gnathostoma* infection from other tissue nematode infection and cutaneous and visceral

gnathostomiasis. This is the first investigation of *Gnathostoma* and gnathostomiasis, concerning the epidemiology in Lao PDR. It has described morbidity and factors influent to the gnathostomiasis in Lao PDR. The result of the study will be used as baseline data for *Gnathostoma* and gnathostomiasis in the country.

4.12 Variables

4.12.1 Independent variables:

- Age
- Sex
- Ethnicity
- Religion
- Occupation
- Educational level of familial head
- Educational level of parents
- Educational level of individual subject
- Habit of raw eating

4.12.2 Extraneuos variables

- Infection rate of advanced third stage larvae within 2nd intermediate hosts
- Infection rate of *Gnathostoma* spp. in definitive hosts
- Accuracy of diagnostic techniques.

4.12.3 Dependent variables:

- Gnathostomiasis.

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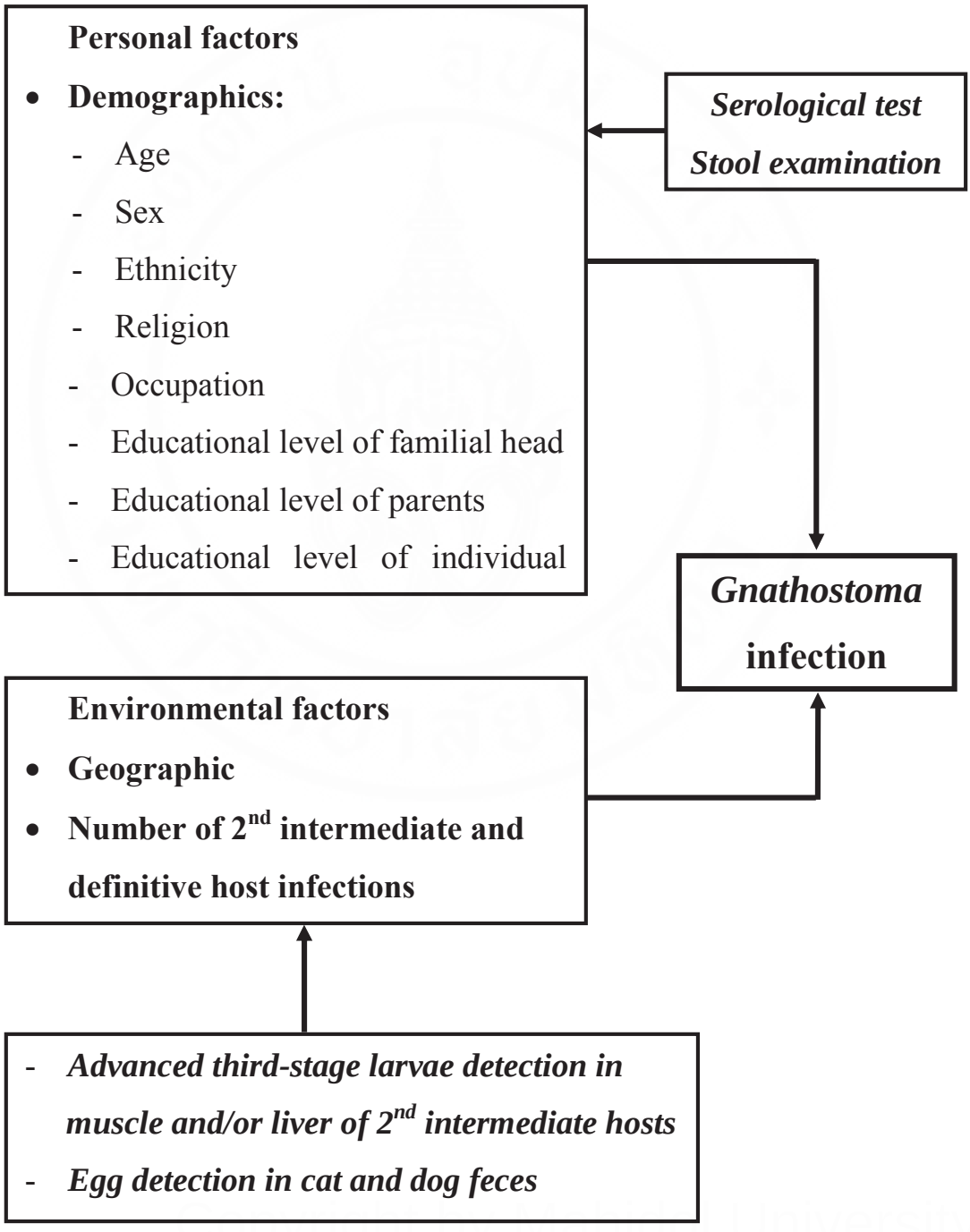
4.13 Conceptual framework

Conceptual framework showing the personal and environmental factors related to *Gnathostoma* infection.



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CHAPTER V
RESULTS

5.1 Preliminary survey for gnathostomiasis cases

Five provinces were performed a preliminary survey to search the gnathostmiasis cases as a hospital-based study. A total of 85 suspected cases were blood collected from the hospitals of each province. In Luangprabang and Savannakhet province had no suspected case of gnathostomiasis and no serum sample available for examination. However, there were 3, 9 and 2 sero-positive against GsAL3 antigen in Bokeo, Vientiane Capital and Champasack province, respectively (Table 5.1).

Table 5.1 Sera analysis of preliminary survey for gnathostomiasis cases

Provinces	No. of examined (Female/Male)	No. of positive (Female/Male)
Bokeo	27 (19/8)	3 (2/1)
Luangprabang	0	0
Vientiane Capital	46 (21/25)	9 (7/2)
Savannakhet	0	0
Champasack	12 (10/2)	2 (1/1)
Total	**85 (50/35)**	**14 (10/4)**

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In the north, no suspected cases were found in the hospital records from Bokeo and Laungprabang. We received 27 donated serum samples from Houeixay Hospital, Bokeo Province, and found 3 sero-positive samples for gnathostomiasis. One of the positive case lives in the nearest village, Phibounthong, hence this site was selected in the north.

In the central, there were several cases reported in Naxon Hospital, Vientiane Capital, and 9 of the serum samples from this hospital were positive with gnathostome antibody. Naxon village was selected as study site in the Central.

In the south, there were cases recorded at Phonthong Hospital, Champasack Province, and serum samples from the hospital were positive with gnathostome antibody. According to suggestion of Phonthong Hospital Director, Nongtearnoy Village was selected as study site in the south.

5.2 Study population

A total of 420 (140/each village) randomly selected participants were interviewed for socio-demography as shown in Table 5.2. Of these, 224 (53.3%) were females. The majority (402, 95.7%) were Laoloums and only 18 (4.3%) were Laotheungs, respectively. The mean age of all participants was 37.6 years old with the minimum and maximum range of 10 to 70 years old. The majority (327, 77.9%) of participants were agriculturists.

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Table 5.2 Characteristics of the randomly selected population in three villages

	Phibounthong[a] n (%)	Naxon[b] n (%)	Nongtearnoy[c] n (%)	Total n (%)
Age (years)				
Mean	35.8	38.7	38.1	37.6
Age range	10-70	10-70	11-68	10-70
Sex				
Male	61 (43.6)	61 (43.6)	74 (52.9)	196 (46.7)
Female	79 (56.4)	79 (56.4)	66 (47.1)	224 (53.3)
Ethnic group				
Laoloum	122 (87.1)	140 (100)	140 (100)	402 (95.7)
Laotheung	18 (12.9)	0 (0.0)	0 (0.0)	18 (4.3)
Educational level				
Illiterate	20 (14.3)	7 (5.0)	17 (12.1)	44 (10.5)
Primary school	71 (50.7)	53 (37.9)	80 (57.1)	204 (48)
Secondary school	23 (16.4)	28 (20.0)	27 (19.3)	78 (18.6)
High school	9 (6.4)	30 (21.4)	5 (3.6)	44 (10.5)
Bachelor's degree	2 (1.4)	2 (1.4)	0 (0.0)	4 (1.0)
Studying*	15 (10.7)	20 (14.3)	11 (7.8)	46 (10.9)
Occupation				
Agriculturist	113 (80.7)	90 (64.3)	124 (88.6)	327 (77.9)
Employee	2 (1.4)	5 (3.6)	0 (0.0)	7 (1.7)
Housewife	2 (1.4)	4 (2.9)	0 (0.0)	6 (1.4)
Merchant	4 (2.9)	2 (1.4)	0 (0.0)	6 (1.4)
Government employee	3 (2.1)	19 (13.5)	5 (3.6)	27 (10.8)
Student	15 (10.7)	20 (14.3)	11 (7.8)	46 (10.9)

* Students who are studying in primary or secondary or high school.

a the village situates in Houeixay District, Bokeo Province, northern part of Lao PDR.

b the village situates in Parkngumg District, Vientiane Capital, central part of Lao PDR.

c the village situates in Phonthong District, Champasack Province, southern part of Lao PDR.

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A total of 172 voluntary participants were also examined for serum analysis. The age and gender of voluntary participants were recorded and shown in Table 5.3.

Table 5.3 Characteristics of voluntary participants in three villages

Villages (n)	Voluntary participants	
	Female/Male	Age range (Mean)
Phibounthong (46)	22/24	11 - 70 (30.1)
Naxon (82)	38/44	12 - 68 (34.8)
Nongtearnoy (44)	26/18	11 - 69 (33.9)
Total (172)	**86/86**	**11 - 70 (33.3)**

5.3 Prevalence of *Gnathostoma* infection

5.3.1 Antibody detection

The sera of randomly selected (420) and voluntary (172) participants were examined for the presence of *Gnathostoma*-specific antibody using immunoblot technique and the results were summarized in Table 5.4. Among 420 randomly selected participants, 125 (29.8%) were found to be sero-positive, and among 172 voluntary participants 25 (14.5%) were sero-positive. As the overall results, 150 of 592 sera were judged as having specific antibody. Analysis on individual provinces demonstrated the highest serum-positive rate of 47.1% in Naxon, followed by 38.6% in Nongtearnoy. Immunoblotting patterns of participants were shown in Figure 5.1. Seroprevalence in Phibounthong was as low as 3.6%, which the seroprevalence distribution in three provinces of Lao PDR have been demonstrated in Figure 5.2.

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Table 5.4 Sero-prevalence of *Gnathostoma* infection in villages

Villages	Randomly selected participants		Voluntary participants		Total	
	No. exam.	No. positive n (%)	No. exam.	No. positive n (%)	No. exam.	No. positive n (%)
Phibounthong	140	5(3.6)	46	1(2.2)	186	6(3.2)
Naxon	140	66(47.1)	82	12(14.6)	222	78(35.1)
Nongtearnoy	140	54(38.6)	44	12(27.3)	184	66(35.9)
Total	**420**	**125(29.8)**	**172**	**25(14.5)**	**592**	**150(25.3)**

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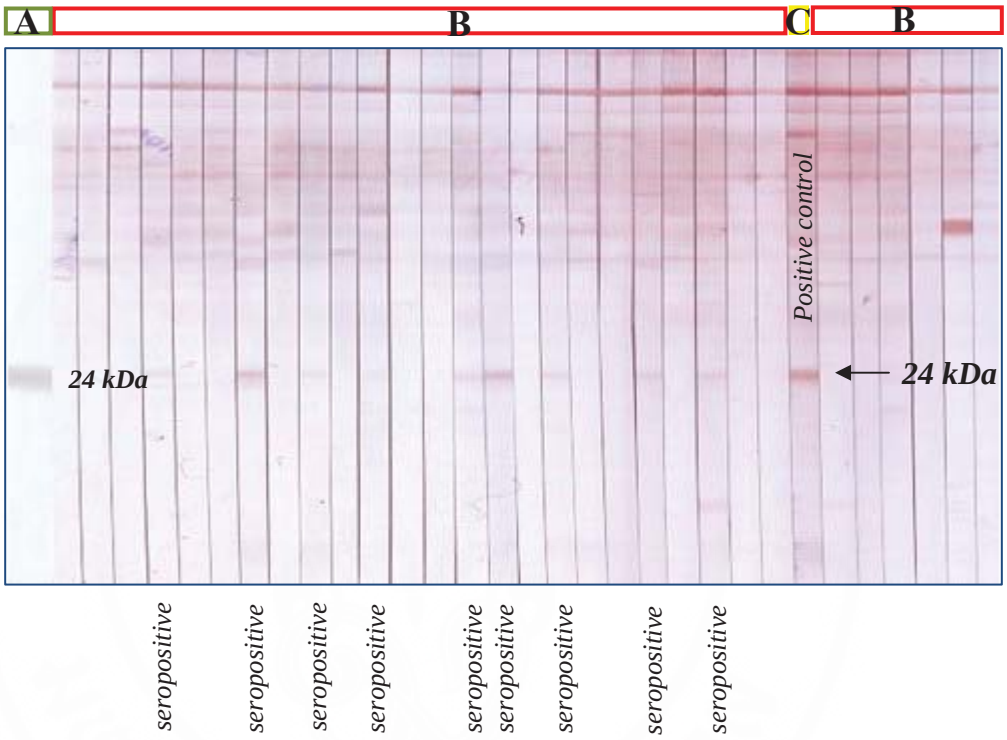

Figure 5.1 The immunoblot technique for diagnosis of human gnathostomiasis.

Lane A: mAb specific to *G.spinigerum* (24 kDa).

Lane B: Serum samples from the Lao participants.

Lane C: Positive control (Thai gnathostomiasis patients).

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Map of Lao PDR

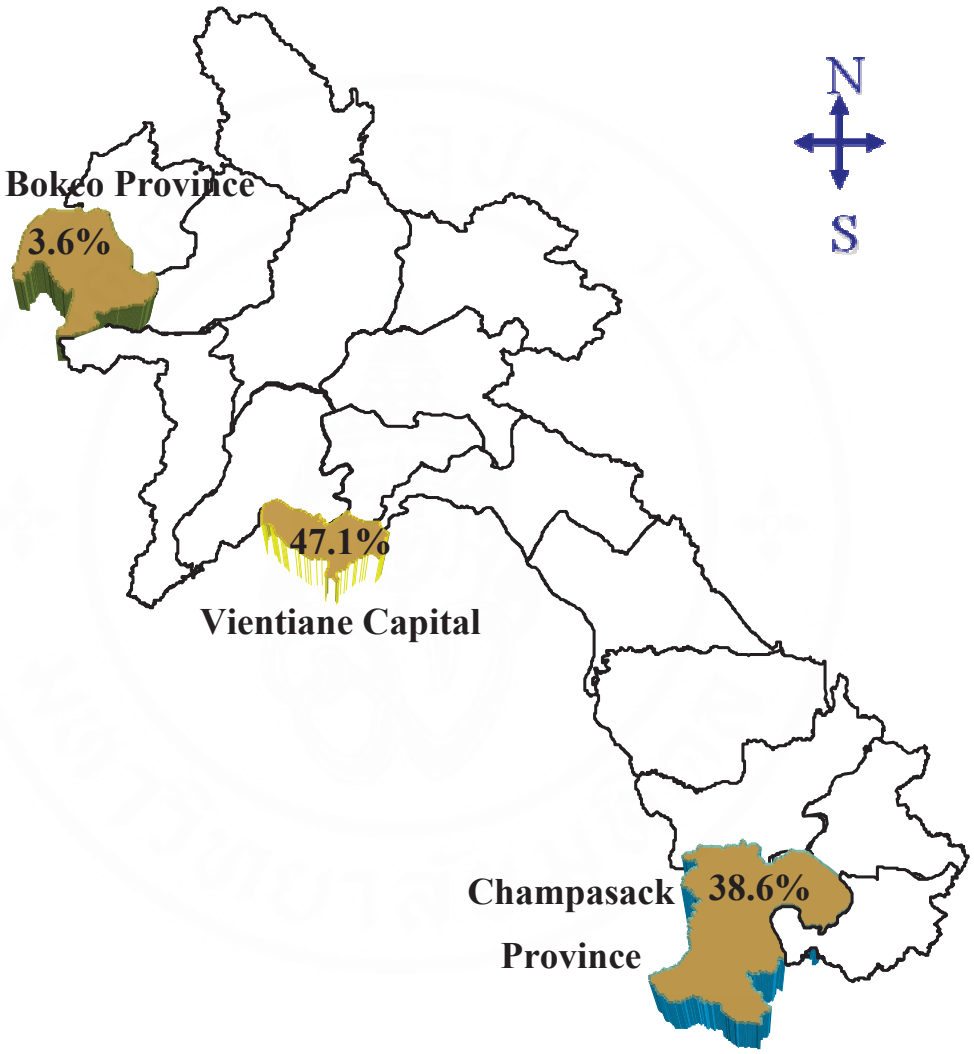

Figure 5.2 Sero-prevalence distribution of *Gnathostoma* infection in three provinces of Lao PDR**.**

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5.3.2 Acute gnathostomiasis

During investigation in Naxon village, we found an active male case of probable gnathostomiasis, age of 32. The patient had a mobile pitting swelling with pain first appeared on his right hand and then migrated to armpit, nape and then left hand during the past month. He also had a history of suffering from migrating intermittent swelling on hands one year ago. He denied eating raw/undercooked fish and fermented fish, but occasionally handle/knead raw fish during preparing fermented fish. The clinical finding of left hand swelling is shown in Figure 5.3.

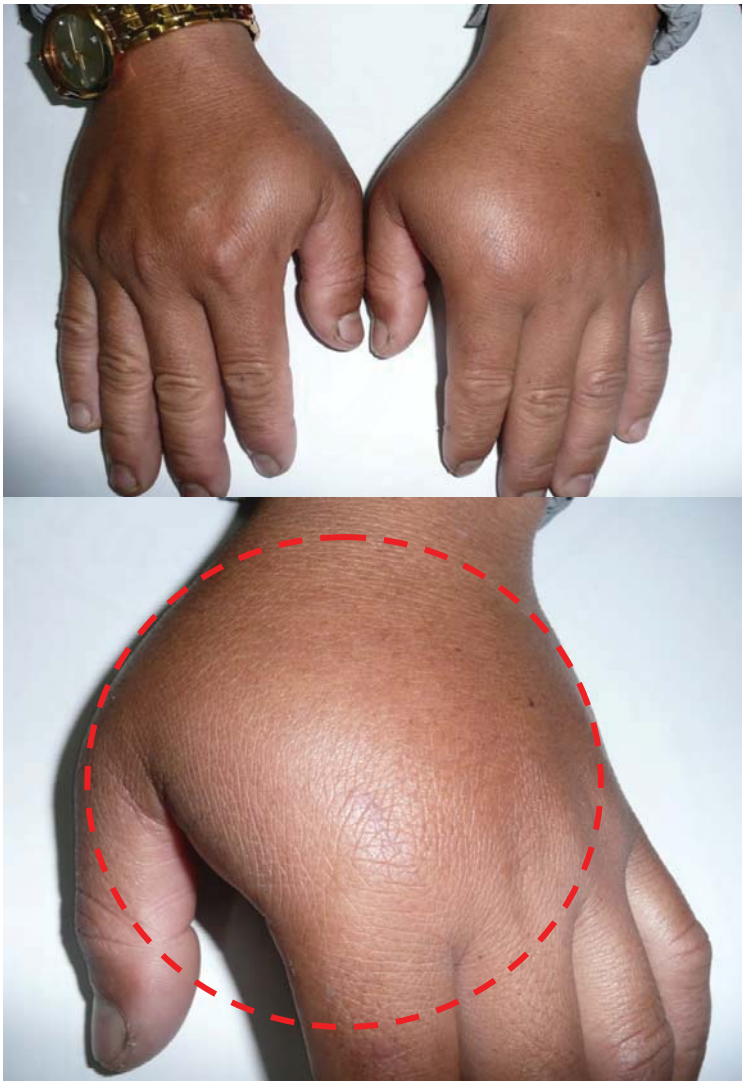

Figure 5.3 Left hand mobile intermittent swelling with tenderness of an acute gnathostomiasis patient.

5.4 Risk factor analyze for *Gnathostoma* infection

5.4.1 Habit consuming factors analysis in three villages

The habit of food consumption of 420 randomly selected participants was interviewed by closed questions. The result showed that raw/undercooked fish consuming was common among three villages, 79.3%, 80.0% and 92.9% in Phibounthong, Naxon and Nongtearnoy, respectively. While, there was no eating habit for raw catfish, swamp eel, frog and chicken or bird among the participants. Raw snake-head consumers were only found in Phibounthong village 77 (55%) as called "Koi soi pa khoo", but no found seropositive with GsAL3 antigen among them. Table 5.5 demonstrated the proportion of raw/undercooked food consuming.

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Table 5.5 Proportion of raw/undercooked food consumption in villages

Raw/undercooked food	Phibounthong n (%)	Naxon n (%)	Nongtearnoy n (%)	Total n (%)
raw/undercooked fish				
Yes	111 (79.3)	112 (80.0)	130 (92.9)	353 (84.0)
No	29 (20.7)	28 (20.0)	10 (7.1)	67 (16.0)
raw/undercooked fermented fish				
Yes	117 (83.6)	122 (87.1)	139 (99.3)	378 (90.0)
No	23 (16.4)	18 (12.9)	1 (0.7)	42 (10)
raw chicken or bird				
Yes	0	0	0	0
No	140 (100)	140 (100)	140 (100)	420 (100)
raw swamp eel				
Yes	0	0	0	0
No	140 (100)	140 (100)	140 (100)	420 (100)
raw snake-head fish				
Yes	77 (55.0)	0	0	77 (18.3)
No	63 (45.0)	140 (100)	140 (100)	343 (81.7)
snake-head fish				
Yes	136 (97.1)	139 (99.3)	140 (100)	415 (98.8)
No	4 (2.9)	1 (0.7)	0	5 (1.2)
catfish				
Yes	137 (97.9)	138 (98.6)	140 (100)	415 (98.8)
No	3 (2.1)	2 (1.4)	0	5 (1.2)
swamp eel				
Yes	123 (87.9)	99 (70.7)	97 (69.3)	319 (76.0)
No	17 (12.1)	41 (29.3)	43 (30.7)	101 (24.0)
frog				
Yes	135 (96.4)	131 (93.6)	140 (100)	406 (96.7)
No	5 (3.6)	9 (6.4)	0	14 (3.3)
Wild animals (raw wild boar, deer)				
Yes	58 (41.4)	102 (72.9)	2 (1.4)	162 (38.6)
No	82 (58.6)	38 (27.1)	138 (98.6)	258 (61.4)
Drink unboiled water				
Yes	133 (95.0)	127 (90.7)	140 (100)	400 (95.2)
No	7 (5.0)	13 (9.3)	0	20 (4.8)

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5.4.2 Raw/undercooked fish consumption

The consumption of raw/undercooked fish was common in all three studied villages. In total, 353 (84%) and 378 (90%) out of 420 randomly selected participants have a custom of consuming raw/undercooked fish and fermented fish, respectively. There are several raw/undercooked fish dishes such as “Laab pa” (minced raw fish mixed with spice and chili served with vegetable); “Koi pa” (pieces of raw fish mixed with spice and chili served with vegetable); “Koi loiloum” (alive small fish mixed with spicy sauce and chili). As the fermented fish dishes, the following recepies are common; “Som pa khoo” (raw snake-head fish meat mixed with spice were packed in banana leaf and kept for a few days), “Pa dek” (a combination of many types of fish mixed with rice bran and salt is put into a pot and kept for a few months or years), “Som pa noi (many types of raw small fish mixed with spice put in a pot were kept in the pot for a few days). Consumption of raw snaked-head fish, cat fish, frog and swamp eel was not common in the study sites, except for the raw snake-head fish dish called “Koi soi pa khoo” in Phibounthong Village, Bokeo Province. The proportion of the people consuming raw/undercooked or fermented fish in each village is shown in Table 5.6.

Table 5.6 Proportion of raw/undercooked foods consumers

Villages	Fish		Wild animals (raw wild boar, deer)
	Raw/undercooked	Raw fermented	
Phibounthong	111(79.3)	117(83.6)	58(41.4)
Naxon	112(80.0)	122(87.1)	102(72.9)
Nongtearnoy	130(92.9)	139(99.3)	2(1.4)
Total	**353(84.0)**	**378(90.0)**	**162(38.6)**

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5.4.3 Bivariate and multivariable analysis of some potential risk factors for *Gnathostoma* infection

A total of 420 randomly selected participants were interviewed about the long practical habit on raw fish handling/kneading for detecting some potential risk factors related to *Gnathostoma* infection. Some kinds of fermented fish preparing process take time approximately more than 30 minutes for kneading, such as fermented snake-head fish called "Som Pa Khoo". Of these, 42 (30%); 17 (12.1%) and 2 (1.4%) stated that the practical habit on raw fish handling/kneading in Naxon, Phibounthong and Nongtearnoy village, respectively. By bivariate analysis screening, the gender, and the consumption of snake-head fish, catfish and frog were excluded from the potential risk factors. The overall data showed the close association of seropositivity to the following factors; raw/undercooked fish, raw fermented fish, raw wild animal consuming, cat or dog domestication, know about gnathostomiasis, raw fish handling/kneading and the history of suffering from migrating intermittent swelling on the body. For the data of individual village was demonstrated the relationship significant of raw/undercooked fish, raw fermented fish, eat swamp eel, raw wild animal consuming, raw fish handling/kneading and report of suffering from migrating intermittent swelling in Naxon village while has only raw/undercooked fish consumption was related to *Gnathostoma* antibody positive participants in Nongtearnoy Village (Table 5.7).

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Table 5.7 Bivariate analysis of some potential risk factors for *Gnathostoma* infection in villages

Potential risk factors	Phibounthong	Naxon	Nongtearnoy	Total
Eat raw/undercooked fish				
Yes/yes with Gn. Ab. Positive (%)	111/5 (4.5)	112/61 (54.5)	130/54 (41.5)	353/120 (33.9)
No with Gn. Ab. Positive	0	5	0	5
	P=0.583 [b]	*P*=0.001 [a]	*P*=0.009 [a]	*P*<0.001 [a]
Eat raw/undercooked fermented fish				
Yes/yes with Gn. Ab. Positive (%)	117/5 (4.3)	122/63 (52.6)	139/54 (38.8)	378/122 (32.3)
No with Gn. Ab. Positive	0	3	0	3
	P=0.591 [b]	*P*=0.006 [a]	*P*=1.000 [b]	*P*=0.001 [a]
Eat snake-head fish				
Yes/yes with Gn. Ab. Positive (%)	136/5 (3.7)	139/66 (47.5)	140/54 (38.6)	415/125 (30.1)
No with Gn. Ab. Positive	0	0	0	0
	P=1.000 [b]	*P*=1.000 [b]	-	*P*=0.328 [b]
Eat catfish				
Yes/yes with Gn. Ab. Positive (%)	137/5 (3.6)	138/66 (47.8)	140/54 (38.6)	415/ 125 (30.1)
No with Gn. Ab. Positive	0	0	0	0
	P=1.000 [b]	*P*=0.498 [b]	-	*P*=0.328 [b]
Eat swamp eel				
Yes/yes with Gn. Ab. Positive (%)	123/4 (3.3)	99/53 (53.5)	97/40 (41.2)	319/97 (30.4)
No with Gn. Ab. Positive	1	13	14	28
	P=0.482 [b]	*P*=0.019 [a]	*P*=0.330 [a]	*P*=0.607 [a]
Eat frog				
Yes/yes with Gn. Ab. Positive (%)	135/5 (3.7)	131/65 (49.6)	140/54 (38.6)	406 /124 (30.5)
No with Gn. Ab. Positive	0	1	0	1
	P=1.000 [b]	*P*=0.036 [b]	-	*P*=0.060 [a]
Wild animal (raw wild boar, deer)				
Yes/yes with Gn. Ab. Positive (%)	58/2 (3.4)	102/58 (56.9)	0	162/60 (37.0)
No with Gn. Ab. Positive	3	8	54	65
	P=1.000 [b]	*P*<0.001 [a]	-	*P*=0.010 [a]
Have cat and/or dog				
Yes/yes with Gn. Ab. Positive (%)	115/4 (3.5)	88/40 (45.5)	38/10 (26.3)	241/54 (22.4)
No with Gn. Ab. Positive	1	26	44	71
	P=1.000 [b]	*P*=0.603 [a]	*P*=0.069 [a]	*P*<0.001 [a]
Know about gnathostomiasis				
Yes/yes with Gn. Ab. Positive (%)	0	128/57 (40.7)	0	12/9 (100)
No with Gn. Ab. Positive	5	9	54	116
	-	*P*=0.043 [a]	-	*P*=0.001 [a]

Handling/Kneading raw fish*				
Yes/yes with Gn. Ab. Positive (%)	17/1 (5.9)	42/27 (64.3)	2/0 (0)	61/28 (45.9)
No with Gn. Ab. Positive	4	39	54	97
	P=0.482 [b]	*P*=0.008 [a]	*P*=0.523 [b]	*P*=0.003 [a]
Suffering from intermittent migratory swelling				
Yes/yes with Gn. Ab. Positive (%)	2/0 (0)	23/16 (69.6)	3/3 (100)	28/19 (67.9)
No with Gn. Ab. Positive	5	50	51	106
	-	*P*=0.018 [a]	*P*=0.055 [b]	*P*<0.001 [a]
Got worm(s) from your skin/wound				
Yes/yes with Gn. Ab. Positive (%)	0	3/2 (66.7)	0	3/2 (66.6)
No with Gn. Ab. Positive	5	64	54	123
	-	*P*=0.602 [b]	-	*P*=0.212 [b]
Suffering from itchy swelling on eye(s)/eyelid(s)				
Yes/yes with Gn. Ab. Positive (%)	0	2/2 (100)	0	2/2 (100)
No with Gn. Ab. Positive	5	64	54	123
	-	*P*=0.220 [b]	-	*P*= 0.088 [b]

a: Pearson's Chi-square test

b: Fisher's exact test

**: Fermented fish preparation process*

The result of the multivariable logistic regression analysis revealed that age, cat or dog domestication, having knowledge about gnathostomiasis was independently related to the risk of sero-positivity. The sero-prevalence increased along with the age. The adults aged 31-40 and 41-50 years had the same 5.07 fold higher risk for *Gnathostoma* infection (*P*=0.005 and 0.006, respectively). Subsequently, the aged over 50 years had an 8.47 fold increased risk than that lower aged (*P*<0.001). Randomly selected participants who reported raw/undercooked fish consuming had a 4.23 fold higher risk of *Gnathostoma* infection compared whom was not raw/undercooked fish practice (*P*=0.042). Report of suffering from migrating intermittent swelling and gender did not assist to the risk of *Gnathostoma* infection (*P*=0.071 and 0.603, respectively) within this study (Table 5.8).

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Table 5.8 Multivariable analysis of selected risk factors for *Gnathostoma* infection, according to variables significant ($P<0.05$) in bivariate analysis)

Potential risk factors	Naxon Village		Nongtearnoy Village		Total	
	Odds ratio (*P*-value)	95% CI	Odds ratio (*P*-value)	95% CI	Odds ratio (*P*-value)	95% CI
Age group						
<20	1.00		1.00		1.00	
21-30	S/N	S/N	1.69 (0.605)	0.22 - 12.65	1.34 (0.657)	0.36 - 4.97
31-40	S/N	S/N	3.78 (0.144)	0.63 - 22.59	5.07 (0.005)	1.63 - 15.85
41-50	S/N	S/N	2.57 (0.317)	0.41 - 16.25	5.07 (0.006)	1.59 - 16.09
>50	S/N	S/N	9.28 (0.015)	1.54 - 56.07	8.47 (<0.001)	2.74 - 26.14
Sex						
Male	1.00		1.00		1.00	
Female	1.15 (0.792)	0.41 - 3.26	0.39 (0.060)	0.15 - 1.04	0.87 (0.603)	0.53 - 1.45
Eat raw/undercooked fish						
No	1.00		1.00		1.00	
Yes	2.67 (0.340)	0.36 - 19.97	2.53 (0.333)	0.27 – 17.95	4.23 (0.042)	1.05 - 17.05
Eat raw/undercooked fermented fish						
No	1.00				1.00	
Yes	2.40 (0.554)	0.13 - 43.61	N/S	N/S	2.31 (0.388)	0.34 - 15.53
Eat swamp eel						
No	1.00		1.00			
Yes	1.98 (0.288)	0.56 - 6.98	0.76 (0.643)	0.25 - 2.34	N/S	N/S
Eat wild animal (raw swine, deer)						
No	1.00				1.00	
Yes	2.93 (0.120)	0.75 - 11.35	N/S	N/S	1.23 (0.447)	0.72 - 2.12

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Table 5.8 Multivariable analysis of selected risk factors for *Gnathostoma* infection, according to variables significant (*P*<0.05) in bivariate analysis) (cont.)

Potential risk factors	Naxon Village		Nongtearnoy Village		Total	
	Odds ratio (*P*-value)	95% CI	Odds ratio (*P*-value)	95% CI	Odds ratio (*P*-value)	95% CI
Eat frog						
No	1.00					
Yes	5.23 (0.192)	0.44 - 62.91	N/S	N/S	N/S	N/S
Have cat and/or dog						
Yes					1.00	
No	N/S	N/S	N/S	N/S	0.39 (<0.001)	0.24 - 0.66
Know about gnathostomiasis						
No	1.00				1.00	
Yes	0.07 (0.028)	0.01 - 0.74	N/S	N/S	0.11 (0.030)	0.01 - 0.80
Handling/Kneading raw fish*						
No	1.00				1.00	
Yes	1.91 (0.213)	0.69 - 5.34	N/S	N/S	1.69 (0.122)	0.87 - 3.27
Suffering from migrating intermittent swelling						
No	1.00				1.00	
Yes	0.97 (0.970)	0.25 - 3.79	N/S	N/S	2.64 (0.071)	0.92 - 7.58

N/S: *Not significant in Pearson's Chi-square or Fisher's exact test.*, **: Fermented fish preparation process*

S/N: *Small number (the model cannot calculate)*

5.4.4 Clinical symptoms and *Gnathostoma* antibody detected cases among the three studied villages

The proportion of the participants who have/had the history of suffering from intermittent migratory swelling were found to be 28/420 (6.7%) and the majority of them (23/28, 82.1%) were the residents in Naxon, central Laos (Table 3). Among those 28 participants having a history of suspected symptoms of gnathostomiasis, 19 cases (67.9%) were sero-positive, suggesting their recent infection with the parasite. In contrast, sero-positive rates of asymptomatic participants was 37.1%.

Table 5.9 Relationship between sero-positivity and the case histories of skin lesions

Clinical symptoms*	Sero-positive/No. exam. (%)			Total
	Phibounthong	Naxon	Nongtearnoy	
No	5/138	50/117	51/137	106/392 (37.1)
Yes	0/2	16/23	3/3	19/28 (67.9)
Total	**5/140**	**66/140**	**54/140**	**125/420 (29.8)**

* History of suffering from intermittent migratory swelling

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5.5 Intermediate and definitive hosts survey

5.5.1 Second intermediate host analysis

Five species of second intermediate host and one species of paratenic host were examined for *Gnathostoma* larvae detection. *Gnathostoma* larvae were both obtained from muscle and liver of the hosts. The result of second intermediate and paratenic hosts revealed that snake-head fish *(Channa striata)*, red-tailed snakehead fish *(Channa limbata)*, climbing perch *(Anabas testudineus)*, swamp eel *(Monopterus albus)* and frog *(Rana sp.)* were the intermediate hosts of *G. spinigerum* in these two villages Naxon and Nongtearnoy, while cat fish were all negative. Whereas, very small number of snake-head and red-tailed snakehead fish were obtained from Phibounthong village. Therefore, the overall of 114 snake-head fish, 118 red-tailed snakehead fish, 363 climbing perch, 154 swamp eel and 97 frog were 4.4%, 2.5%, 2.8%, 20.1% and 7.2% positive respectively with GsAL3 (Figures 5.4-6). This study reports the first recorded of *Channa limbata* (red-tailed snakehead fish) to be as natural second intermediate hosts. The data of individual by village was shown in Table 5.10.

Table 5.10 Prevalence of GsAL3 in intermediate hosts in three villages

Intermediate hosts	Phibounthong No. +ve/exam. (%)	Naxon No. +ve/exam. (%)	Nongtearnoy No. +ve/exam. (%)	Total No. +ve/exam. (%)
Snake-head fish *(Channa striata)*	0/15 (0)	4/85 (4.7)	1/14 (7.1)	5/114 (4.4)
Red-tailed snakehead fish *(Channa limbata)**	0/2 (0)	3/113 (2.7)	0/3 (0)	3/118 (2.5)
Cat fish *(Clarius batrachus)*	0	0/2 (0)	0/17 (0)	0/19 (0)
Climbing perch *(Anabas testudineus)*	0	6/296 (2)	4/67 (5.9)	10/363 (2.8)
Swamp eel *(Monopterus albus)*	0	27/126 (21.4)	2/28 (7.1)	31/154 (20.1)
Frog *(Rana sp.)*	0	1/36 (2.8)	6/61 (9.8)	7/97 (7.2)

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* First record as the natural second intermediate host.

Figure 5.4 *G. spinigerum* advanced-third stage larvae obtained from 2nd intermediate hosts

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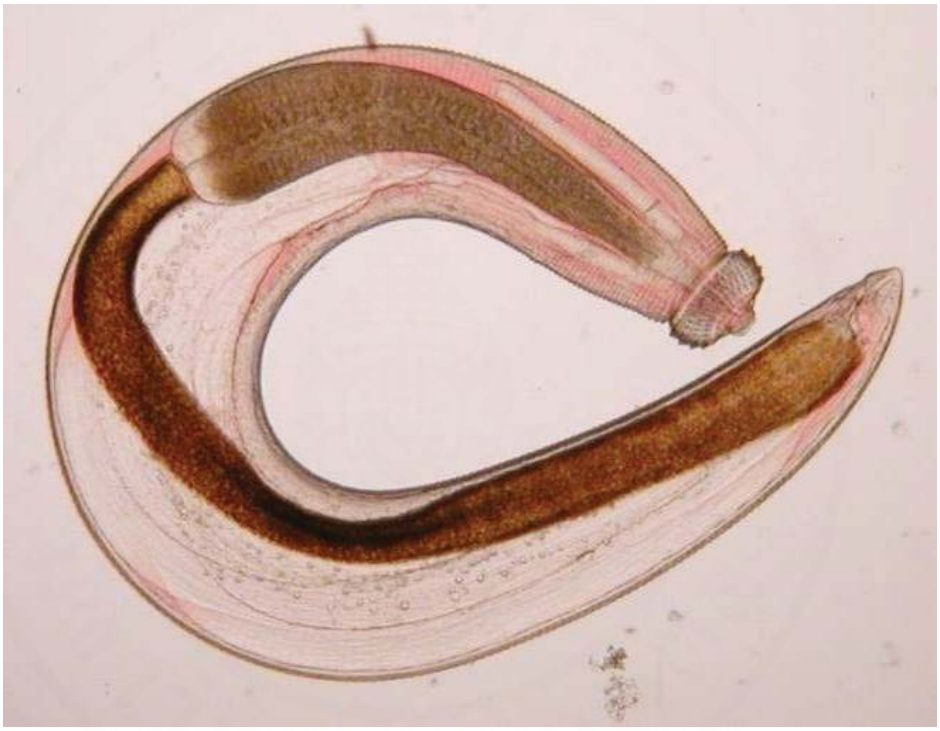

Figure 5.5 *G. spinigerum* advanced-third stage larvae obtained from 2nd intermediate hosts.

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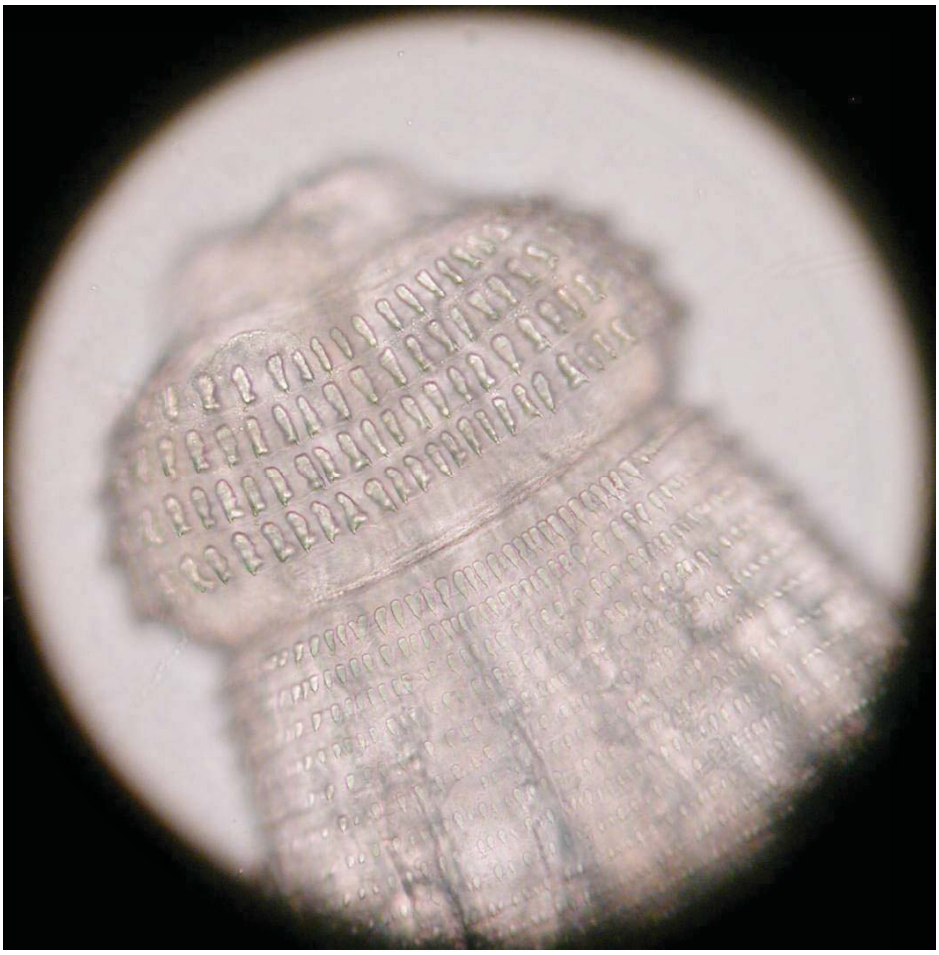

Figure 5.6 The typical cephalic hooklets of *Gs*AL3 obtained from 2nd intermediate hosts

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5.5.2 Definitive host analysis

Dropped fecal samples of dogs were collected from three villages. Among a total of 100 specimens, only 2 positives (2%). There was only dog dropped feces from Nongtearnoy village were positive for *Gnathostoma* eggs. The eggs were identified morphologically as *G. spinigerum* eggs (Figure 5.7-8). In Table 5.11 showed the numbers of dropped fecal samples of dog were examined and positive for *Gnathostoma* eggs.

Table 5.11 Numbers of fecal samples of dog dropped positive for *Gnathostoma spinigerum* eggs in villages

Villages	No. examined	No. positive	% positive
Phibounthong	34	0	0
Naxon	52	0	0
Nongtearnoy	14	2	14.3
Total	**100**	**2**	**2**

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Figure 5.7 *Gnathostoma* eggs obtained from dog's feces in Nongtearnoy Village. A. *Gnathostoma* egg with one cell; B. *Gnathostoma* egg developed into two cells. (Normal saline).

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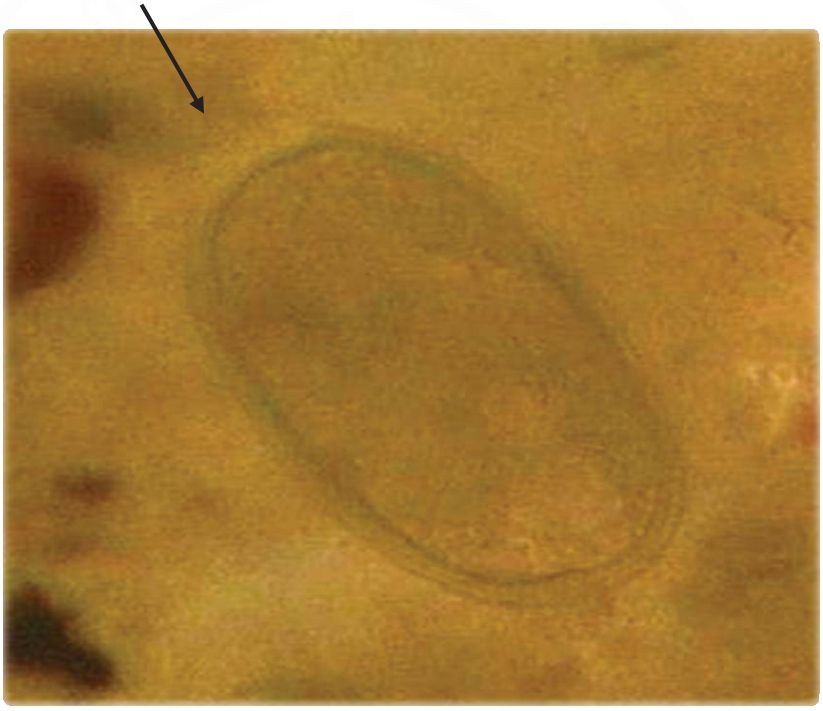

Figure 5.8 *Gnathostoma* eggs obtained from dog's feces in Nongtearnoy Village (Kato-Katz)

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CHAPTER VI
DISCUSSION

This is the first extensive sero-epidemiological survey on gnathostomiasis in Lao PDR using immunoblot technique (Tapchaisri et al., 1991; Dekumyoy et al., 2002; Laummaunwai et al., 2007). A total of 420 randomly selected participants revealed that high prevalence of *Gnathostoma* infection in Laos 29.8%. Overall 592 combined participants were 25.3%, while 14.5% from voluntary participants. Although many studies of *Gnathostoma* have been conducted in Asia, there were not many sero-epidemiological studies. However, in comparison with the sero-epidemiological study in Mexico revealed 34.9% positivity (Camacho et al., 2003), which was higher than ours and *G. binucleatum* was proven pathogen. The results from our study might be not described the exact estimate prevalence of human *Gnathostoma* infection because of human *Gnathostoma* antibody especially IgG could be positive for a few years after worm removal (Morakote et al., 1991b), so that it is likely seen high rate of *Gnathostoma* infection in Naxon and Nongtearnoy village. The prevalence *Gnathostoma* infection was lower than other food-borne zoonotic parasites, especially trematode infections (Kobayashi et al., 2000; Rim et al., 2003; Chai et al., 2005; Sayason et al., 2007; 2009).

Although *Gnathostoma* antibody detection rate in Naxon and Nongtearnoy village were both high, historical report of gnathostomiasis clinical symptoms in Nongtearnoy Village was very low. The reason might be visceral gnathostomiasis due to non suffering from mobile intermittent swelling or there are parasitic morphological variation concerning which serve their special characteristic of clinical showing.

Risk factors analysis was based on the closed questions; the overall data showed significant association between raw/undercooked fish consumption to sero-positive participants. Probable explanation consist of eating behavior of villagers

prefer to consume many types of raw/undercooked and fermented-fish which is the intermediate hosts of *Gnathostoma* spp especially in two villages Naxon and Nongtearnoy village. These results may be describe the important potential sources of *Gnathostoma* transmission within our population and support other studied documents as association between raw/undercooked fish consumption and human *Gnathostoma* infection (Daengsvang, 1980; Miyazaki, 1991; Camacho et al., 2003; Nawa et al., 2004; Rojekittikhun, 2005). Moreover, percutaneous transmission of GsAL3 have been reported from the experimental study (Daengsvang, 1980) so that skin transmission might be occurred in human within our population, but no significant association for longtime practice raw fish handling/kneading in Naxon Village. However, raw fish handling/kneading practice was common in Naxon, this observation should be noted for further study. In this study, sero-positive rate increased with age. It might be an accumulation of infection over time due to continuous exposure or long persistent infection. The sero-prevalence in Phibounthong Village, Bokeo Province, was low although raw snake-head fish consumption was common in that area and all five sero-positive cases have never gone outside from the province. Low sero-prevalence in Phibounthong may be due to low natural host's infection with Gnathostome larvae in this village. Although, the numbers of intermediate hosts collected from Phibouthong were too small, snake-head fish were negative for *Gnathostoma* larvae, the life cycle of *Gnathostom*a spp. have many definitive and intermediate hosts, especially second intermediate and paratenic hosts such as chickens, birds, amphibians, reptiles and mammals (Daengsvang, 1980; Miyazaki, 1991). Therefore, these hosts may be potential sources of transmission even there was no report of consumption these hosts in raw from all participants. Cats and dogs are definitive hosts for *Gnathostoma* spp. (Daengsvang, 1980; Miyazaki, 1991) and these hosts were relative link to sero-positive cases in our study. Especially, dogs from Nongtearnoy, most of them lived a half raised by people. Frequently, they have to find their food in the nature by themselves.

All hosts were collected from natural environment such as canal, pool, rice field, stream, swamp and marsh in or around the studied villages. Our results show that the intermediate and definitive hosts were infected with *Gnathostoma* spp. in two

villages, Naxon and Nongtearnoy, while there were negative in Phibounthong Village. Because of intermediate hosts were collected in Phibounthong in dry season, March, so that we could collect very small numbers of not enough species for examination. Whereas, the intermediate hosts were collected twice in the rainy season (August) in Nongtearnoy and the beginning of dry season and rainy season (December and September) in Naxon village. The report from Thailand showed 16 species of fish were found GsAL3, which swamp eel *(Fluta alba)* was high prevalence of 80% (Daengsvang, 1980). However, the infection rate for *Gnathostoma* larvae in swamp eels was variable from 40 to 100% (Setasuban et al., 1991; Rojekittikhun et al., 1998). In this study, we examined four species of fish which showed lower prevalence of GsAL3 infection than those being reported in Thailand previously (Daengsvang, 1980; Setasuban et al., 1991; Rojekittikhun et al., 1998). Moreover, in our study *Channa limbata* (Red-tailed snakehead fish) was reported new species for natural second intermediate hosts. *Channa limbata* has been not documented from other authors so far (Daengsvang, 1980; Rojekittikhun et al., 1989; 1998; Setasuban et al., 1991; Rojekittikhun, 2005).

Until now there is no report on the prevalence of gnathostomiasis in dogs in Lao PDR; so that this study is the first in this country. In Thailand, *Gnathostoma* infection in dogs is variable depending on time and localities. Two reports in 1962 showed a prevalence rate of 1.6% in fecal samples of dog in Bangkok and Thonburi and 10% from dog in Bangkok (Ito et al., 1962; Sirisumpan, 1962). Furthermore, 1.2% in fecal samples of dogs was positive with *G. spinigerum* eggs in Nakhon Nayok Province (Rojekittikhun, 2000). Report from the northeastern part of Thailand demonstrated 4.1% of dogs were Gnathostome egg positive. In these previous reports both fecal samples and stomachs of dogs were investigated to detect *G. spinigerum* eggs and adult worms. More recently, stomachs and intestines of 200 stray dogs were examined in 1998, but were negative for Gnathostome worms (Rojekittikhun, 2005). In this study 14.2% of fecal samples of dogs were positive for *G. spinigerum* eggs in only Nongtearnoy Village, Champasack Province. Surprisingly, fecal samples from Naxon Village were negative for Gnathostome eggs, where the seroprevalence for *Gnathostoma* infection in human was 47.1% and Gnathostome larvae infection rate of

21.4%, 4.7% and 2.8% in swamp eels, snake-head fish and frogs, respectively. One of the possible explanations for this may be due to the annual fluctuation of the prevalence of gnathostomiasis in dogs (Manning et al., 1969), but need seasonal variation study of animal hosts for confirmation this explanation. Another reason is that Naxon Village itself is not an endemic area, but the disease is endemic surrounding villages. Naxon Village shares the rice field area, pool, swamp and marsh for agriculture with other villages. Especially, Nong Han swamp is shared with many villages such as Naxon and Don Hai village. In addition, fecal sample of the same hosts may be collected repeatedly in our study so that the prevalence of dogs unlikely with *Gnathostoma* was seen be high in Naxon Village.

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CHAPTER VII
CONCLUSIONS AND RECOMMENDATIONS

7.1 Conclusions

In conclusion, the sero-prevalence of human *Gnathostoma* infection using immunoblot for detecting class-specific antibody IgG against 24 kDa antigen was defined as positive case seemed to be appropriate for seroepidemiological studies (Laummaunwai et al., 2007) which our study confirms that statement. This study showed high prevalence of human *Gnathostoma* infection in two villages Naxon and Nongtearnoy village. Raw or undercooked fish consumption was associated to human *Gnathostoma* infection, which is the confirmation of important risk factors for the disease transmission (Daengsvang, 1980; Miyazaki, 1991). Swamp eel and snake-head fish were the important natural hosts to contribute parasite life cycle in the environment. Moreover, the life cycle is maintained in Nongtearnoy village, where dogs are the reservoir host and swamp eels, fish, frogs are the potential source of infection to humans. The morphological confirms the parasite species as *Gnathostoma spinigerum*.

7.2 Recommendations

Further studies on serodiagnosis with questionnaire interview of *Gnathostoma* infection are needed in other epidemiologic setting to determine the nation wide scale, which factors are the most determinants of disease transmission in this country.

More species of natural hosts should be examined to assess the other species of *Gnathostoma* in the country. In addition, the studies for seasonal variation of the *Gnathostoma* infection in various hosts are necessary.

According to the result, public health efforts should focus on prevention of human gnathostomiasis in those endemic areas especially in Naxon and Nongtearnoy Village. Physicians who work in those two infection areas should be awaked of all patients complained with intermittent swelling as gnathostomiasis. Moreover, the villages nearby Naxon and Nongtearnoy village should be further investigated to assess how big is the endemic areas.

Therefore, morphology and molecular studies of the worms in those two villages should be further conducted to characterize their structural and genetic variation which might influence the violence of worms.

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APPENDICES

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APPENDIX A
PARASITE COLLECTION

1% Pepsin-HCl solution

Pepsin	1.00g
HCl	1.00 ml

Distilled water was added to make 100 ml.

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APPENDIX B
PROTEIN DETERMINATION

Reagents

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

This chemical was commercially prepared by PIERCE, USA and stored at -10°C.

2. Coomassie®plus protein assay reagent

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

Protocol

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

2. Prepare unknown samples in required dilution.

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

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

5. Read the absorbance value at 595 nm.

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

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

Reagent

1. Acrylamide: Bis-acrylamide solution

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

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

2. 0.5 M Tris-HCl buffer, pH 6.8

Tris (Hydroxymethyl aminomethane)	6.05 g
DW	50.0 ml

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

3. 1.5 M Tris-HCl buffer, pH 8.8

Tris	18.15 g
DW	50.00 ml

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

4. 10% Sodium dodecyl sulfate (SDS)

SDS	10.0 g
DW	100.0 ml

The solution is kept at room temperature.

5. 10% Ammonium persulfate

Ammonium peroxodisulfate	1.0 g
DW	10.0 ml

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

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

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

7. Sample buffer (x3)

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

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

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

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

The buffer is kept at 4°C.

9. Gradient gel preparation

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

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10. 5% stacking gel

DW	1.50 ml
0.5M Tris-HCl, pH 6.8	0.63 ml
Acrylamide: Bis-acrylamide	0.40 ml
10% SDS	25.00 µl
10%Ammonium persulfate	12.50 µl
TEMED	2.50 µl

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

11. Coomassie brilliant blue stain

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

The staining solution is kept at room temperature.

12. Destaining solution for gel

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

The solution is stored at room temperature.

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APPENDIX D
QUESTIONNAIRE

Questionnaire for interviewing the participants at the study areas of the research on

***Gnathostoma* and gnathostomiasis in three provinces of Lao PDR**

Province:…… …… **District:**…… …… **Village:**…… ……	**CODE OF PARTICIPANT** _____
Interviewer's name:…… ……	***Date:***…… ……

PART 1: DEMOGRAPHIC DATA					
1	Age:_____ (____/____/_____)	[___]	**2**	Sex: 1. Male, 2. Female	[___]
3	Ethnicity: 1. Laolum, 2. Laotheug 3. Laosung, 4. Other:_____	[___]	**4**	Religion: 1. Buddhist 2. Christian 3. Animism 4. Other:_____	[___]
5	Occupation: 1. Agriculturist 2. Fisherman 3. Government official:_____ 4. Employee: _____ 5. Business: _____ 6. Other:_____	[___]	**6**	Educational level: *(if age ≥ 18, skip Q.7, 8)* 1. Illiterate 2. Primary school 3. Secondary school 4. High school 5. Bachelor:_____ 6. Other:_____	[___]
7	Educational level of father: 1. Illiterate 2. Primary school 3. Secondary school 4. High school 5. Bachelor:_____ 6. Other:_____	[___]	**8**	Educational level of mother: 1. Illiterate 2. Primary school 3. Secondary school 4. High school 5. Bachelor:_____ 6. Other:_____	[___]

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9	Do you have cat and/or dog? 1. Yes, 2. No *If No, skip to Q.15*	[___]	**10**	How many cats do you have?_____	[___]
11	How many dogs do you have?_____	[___]	**12**	Have you ever feed cat and/or dog with raw foods? 1. Yes, 2. No, 3. Don't know *If No/Don't know, skip to Q.15*	[___]
13	What kind of raw foods did you Feed cats?_____		**14**	What kind of raw foods did you feed dogs?_____	
PART 2: KNOWLEDGE, ATTITUDE AND PRACTICE					
15	Do you know what gnathostomiasis is? 1. Yes, 2. No *If No, skip to Q.17*	[___]	**16**	If yes describe: _____ _____	
17	How do people get *Gnathostoma* infection? *(can have more than 1 answer)* 1. eat raw/undercooked vegetable 2. eat raw/undercooked fish 3. eat raw/undercooked pork 4. eat raw/undercooked beef, buffalo 5. eat raw/undercooked snake 6. eat raw/undercooked eel 7. eat raw/undercooked chicken, bird 8. skin penetration by larvae 9. other:_____	[___]	**18**	How to prevent *Gnathostoma* infection? *(can have more than 1 answer)* 1. washing hands before and after meal 2. don't eat raw/undercooked fish 3. don't eat raw/undercooked pork 4. don't eat raw/undercooked beef, buffalo 5. don't eat raw/undercooked snake 5. don't eat raw/undercooked eel 7. don't eat raw/undercooked chicken, bird 8. don't use animal component cover skin 9. other:_____	[___]
19	What is the traditional medicine using for abscess, swollen, wound? 1. Know, 2. Don't know *If know*:_____ _____	[___]	**20**	Did you ever apply/cover abscess, swollen, wound with any components of animal? 1. Yes, 2. No *If Yes*:_____ _____	[___]
PART 3: EATING BEHAVIOR					
21	Have you ever eaten raw/undercooked pork? 1. Yes, 2. No *If No, skip to Q.23*	[___]	**22**	What is the name of raw/undercooked pork dishes? Do you like to prepare? _____ _____	
23	Have you ever eaten raw/undercooked beef/buffalo meat?	[___]	**24**	What is the name of raw/undercooked	

	1. Yes, 2. No *If No, skip to Q.25*			beef/ buffalo dishes? Do you like to prepare? _____ _____	
25	Have you ever eaten raw/undercooked chicken and bird meat? 1. Yes, 2. No *If No, skip to Q.27*	[___]	**26**	What is the name of raw/undercooked chicken and bird dishes? Do you like to prepare? _____ _____	
27	Have you ever eaten raw/undercooked fish? 1. Yes, 2. No *If No, skip to Q.29*	[___]	**28**	What is the name of raw/undercooked fish dishes? Do you like to prepare? _____ _____	
29	Have you ever eaten raw/undercooked swamp eel? 1. Yes, 2. No *If No, skip to Q.31*	[___]	**30**	What is the name of raw/undercooked swamp eel dishes? Do you like to prepare? _____ _____	
31	Have you ever eaten raw/undercooked fermented fish? *(Pa deck, Pa som,…)* 1. Yes, 2. No *If No, skip to Q.33*	[___]	**32**	What is the name of raw/undercooked fermented fish dishes? Do you like to prepare? _____ _____	
33	Do you eat snake-headed fish? 1. Yes, 2. No *If Yes*:What is the name of dishes do you like to prepare _____ _____	[___]	**34**	Do you eat swamp eel? 1. Yes, 2. No *If Yes*:What is the name of dishes do you like to prepare _____ _____	[___]
35	Do you eat cat-fish? 1. Yes, 2. No *If Yes*: What is the name of dishes do you like to prepare _____ _____	[___]	**36**	Do you eat frog? 1. Yes, 2. No *If Yes*: What is the name of dishes do you like to prepare _____ _____	[___]
37	Other foods that you eat raw/ undercooked? 1. Yes, 2. No	[___]	**38**	What kind of other fish do people/you in this village prefer to eat	

	If Yes:_____ _____			raw/undercooked? _____ _____	
39	Have you ever drunk unboiled water from stream/pool? 1. Yes, 2. No	[___]	**40**	Have you ever handling/cutting raw fish/meat with wounded hand? 1. Yes, 2. No *If Yes*:_____	[___]
PART 4: RECENT MEDICAL HISTORY (IN THE PAST 4 WEEKS)					
41	Do you have fever/hot body? 1. Yes 2. No	[___]	**42**	Do you have headache? 1. Yes 2. No	[___]
43	Do you have no appetite? 1. Yes 2. No	[___]	**44**	Do you have nausea? 1. Yes 2. No	[___]
45	Do you have vomiting? 1. Yes 2. No	[___]	**46**	Do you have abdominal pain? 1. Yes 2. No *If Yes*:_____ Location:_____	[___]
47	Do you have itchy skin? 1. Yes 2. No *If Yes*:_____ Location:_____	[___]	**48**	Do you have migrating intermittent swollen on your body? 1. Yes 2. No *If Yes*:_____ Location:_____	[___]
49	Do you have migrating intermittent muscle pain? 1. Yes 2. No *If Yes*:_____ Location:_____	[___]	**50**	Do you have creeping eruption? 1. Yes 2. No *If Yes*:_____ Location:_____	[___]
51	Have you ever seen any worms migrate out on you skin/body? 1. Yes 2. No *If Yes*:_____ Location:_____	[___]	**52**	Any other symptoms? 1. Yes 2. No *If Yes*:_____ Location:_____ _____	[___]
PART 5: PAST MEDICAL HISTORY					
53	Had you been suffering from migrating intermittent swollen on your body? 1. Yes 2. No *If Yes*:_____ Location:_____ When: _____	[___]	**54**	Had you been suffering from migrating intermittent muscle pain? 1. Yes 2. No *If Yes*:_____ Location:_____ When:_____	[___]

55	Had you been suffering from creeping eruption? 1. Yes 2. No *If Yes*:_____ Location:_____ When:_____	[___]	**56**	Did you ever get a worm from skin/wound by yourself? What kind of worm is looked like? 1. Yes 2. No *If Yes*:_____ Location:_____ When:_____	[___]
57	Had you been suffering from itchy swollen eyes/eyelid? 1. Yes 2. No *If Yes*:_____ Location:_____ When:_____	[___]	**58**	Any other symptoms? 1. Yes 2. No *If Yes*:_____ Location:_____ When:_____	[___]
PART 6: PHYSICAL EXAMINATION					
59	General status: 1. Good, 2. Not good *If Not good:* _____ When: _____	[___]	**60**	Skin lesion: 1. Normal, 2. Abnormal *If abnormal:* _____ Location:_____ When:_____	[___]
61	Head and Neck: 1. Normal, 2. Abnormal *If abnormal:* _____ Location:_____ When:_____	[___]	**62**	Extremity part: 1. Normal, 2. Abnormal *If abnormal:* _____ Location:_____ When:_____	[___]
63	Abdominal: 1. Normal, 2. Abnormal *If abnormal:* _____ Location:_____ When:_____	[___]	**64**	**OTHER IMPRESSIONS/ INCIDENTAL FINDINGS**:	

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BIOGRAPHY

NAME	Youthanavanh Vonghachack
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DATE OF BIRTH	17 July 1976
PLACE OF BIRTH	Savannakhet Province, Lao PDR
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