A CROSS SECTIONAL INVESTIGATION ON THE OCCURRENCE OF TREMATODE METACERCARIAE IN FRESH WATER CRAB, *Sartoriana spinigera* IN BANGLADESH

A Thesis

By

AL - WASEF

MASTER OF SCIENCE IN PARASITOLOGY

DEPARTMENT OF MICROBIOLOGY AND PARASITOLOGY

SHER-E-BANGLA AGRICULTURAL UNIVERSITY

DHAKA-1207

JUNE, 2020

A CROSS SECTIONAL INVESTIGATION ON THE OCCURRENCE OF TREMATODE METACERCARIAE IN FRESH WATER CRAB, *Sartoriana spinigera* IN BANGLADESH

by

AL - WASEF

Reg. No.: 13 - 05634

A Thesis
Submitted to the Department of Microbiology and Parasitology
Sher-e-Bangla Agricultural University, Dhaka
In Partial Fulfillment of the Requirements
for the degree of

MASTER OF SCIENCE (M.S.) IN PARASITOLOGY

SEMESTER: Jan-Jun/2020

APPROVED BY

Dr. Uday Kumar Mohanta
Supervisor
Chairman and Associate Professor
Department of Microbiology and Parasitology
Sher-e-Bangla Agricultural University

Dr. Anisuzzaman (Aslam)
Co-Supervisor
Professor
Department of Parasitology
Bangladesh Agricultural University

Dr. Uday Kumar Mohanta
Chairman
Department of Microbiology and Parasitology
Sher-e-Bangla Agricultural University

DEPARTMENT OF MICROBIOLOGY AND PARASITOLOGY

Sher-e-Bangla Agricultural University

Sher-e-Bangla Nagar, Dhaka-1207

Memo No: SAU/

CERTIFICATE

*This is to certify that the thesis entitled "**A CROSS SECTIONAL INVESTIGATION ON THE OCCURRENCE OF TREMATODE METACERCARIAE IN FRESH WATER CRAB, Sartoriana spinigera IN BANGLADESH**" submitted to the Faculty of Animal Science & Veterinary Medicine, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of **Master of Science in Microbiology and Parasitology,** embodies the result of a piece of bona fide research work carried out by **Al-Wasef,** Registration No. **13-05634** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.*

I further certify that any help or source of information, received during the course of this investigation has been duly acknowledged.

Dr. Uday Kumar Mohanta
Supervisor
Department of Microbiology and Parasitology
Sher-e-Bangla Agricultural University

Date:

Place: Dhaka, Bangladesh

Dedicated to
My Beloved Grandmother

ACKNOWLEDGEMENT

At the beginning, the author bows the grace and mercy of the "Almighty Allah", the omnipresent, omnipotent and omniscient, who enabled him to complete this thesis.

The author with a sense of respect, expresses his heartfelt gratitude to his Supervisor Dr. Uday Kumar Mohanta, Chairman and Associate Professor, Department of Microbiology and Parasitology, Sher-e-Bangla Agricultural University, Dhaka for his untiring and painstaking guidance, invaluable suggestions, continuous supervision, timely instructions, inspirations and constructive criticism throughout the tenure of research work.

Heartfelt gratitude and profound respect are due to his Co-supervisor Dr. Anisuzzaman, Chairman and Professor, Department of Parasitology, Bangladesh Agricultural University, Mymensingh for his co-operation, constructive criticism, and valuable suggestions for the modification and improvement of the research work.

The author is also grateful to all the staffs of the Department of Microbiology and Parasitology, Sher-e-Bangla Agricultural University, Dhaka for their co-operation. The author deeply owes his whole hearted thanks to all the relatives, friends, well-wishers specially Anup Kumar Adhikary, , for their help and inspiration during the period of the study.

The author takes the opportunity to express his indebtedness and profound respect to his beloved grandmother Banuara Khatun, father Monirul Islam, mother Altafon Nesa, brother Md. Tarikuzzaman, sister Mst Rexona Khatun, uncle Md. Firoz Mahmud, cousin Mst Lovely Akter and Sadia Kader Oshin for their love, blessings, prayers, sacrifices, moral support and encouragement for his study which can never be forgotten.

The author sincerely acknowledges the financial aid from ministry of science and technology as NST fellowship that enable him to complete the research work more smoothly.

The Author

CONTENTS

CONTENTS (CONT'D)

LIST OF FIGURES

LIST OF FIGURES (CONT'D)

LIST OF TABLES

ACRONYMS AND ABBREVIATIONS

ABBREVIATION		FULL MEANING
Cm	=	Centimeter
mm	=	Millimeter
mc	=	Metacercariae
CONT'D	=	Continued
Dr.	=	Doctor
e.g.	=	For example
etc.	=	Et cetera
et al.	=	And others/Associates
HCl	=	Hydrochloric acid
hrs.	=	Hours
i.e.	=	That is
M.S.	=	Master of Science
n	=	Sample number
NO.	=	Number
sp.	=	Single species
spp.	=	Plural species
X	=	Times (Magnification)

LIST OF SYMBOLS

SYMBOLS		FULL MEANING
+	=	Plus
±	=	Plus or minus
<	=	Less than
>	=	Greater than
%	=	Percentage
&	=	And

A CROSS SECTIONAL INVESTIGATION ON THE OCCURRENCE OF TREMATODE METACERCARIAE IN FRESH WATER CRAB, *Sartoriana spinigera* IN BANGLADESH

ABSTRACT

Freshwater crabs are distributed throughout the tropical and sub-tropical regions of the world that are consumed for the purposes of medical and tonic properties, including the treatment of stomach ailments and physical injuries. *Sartoriana spinigera* is an important edible freshwater crab species which play an important role as intermediate host of *Microphallus indicus*. It serves as biological vector for transmission of their infective stages to the final host namely herring gulls, shore-birds, ducks and rodents. We targeted to investigate the prevalence of lung fluke in Bangladesh because *Paragonimus westermani,* the lung fluke has been reported from Northeast India including Assam, Meghalaya, Manipur, Arunachal. During survey, the metacercariae of food borne trematodes were investigated from a total of 261 crabs collected from different geographies of Bangladesh. We could not recover any metacercaria of *Paragonimus* spp. from the examined crab. The metacercariae of *M. indicus* were recovered from the crabs screened in this study. The overall prevalence of *M. indicus* metacercariae was 26% with intensity of 1-63 per crab. The highest (83.33%) prevalence was recorded from Naogaon district, northern part of Bangladesh followed by Sirajgonj (57.5%), Rajshahi (40%), Brahmanbaria (16%), Narsingdi (15%), Faridpur (10%), and Sunamgonj (7%). None of the crabs collected from Rangpur, Thakurgoan, Jashore, Meherpur, Magura, Barguna, Sylhet, Kishorgonj, Cox'sBazar and Bandarban was found to be infected with any microphallid metacearia. Whereas, the highest number of infected crabs were found the in rivers (56%), followed by haor, canal and ditches (5%) and ponds (0%). To the best our knowledge, *M. indicus* is going to be reported for the first time from Bangladesh. Therefore, proper attention should be paid during consumption of crabs.

Keywords: Freshwater crabs, *S. spinigera, M. indicus,* metacercariae, prevalence, Bangladesh

CHAPTER 1

INTRODUCTION

Crabs are the most advanced members of the phylum Arthropoda. True crabs belonging to the order Decapoda under class the Crustacea, and show the greatest size range of all arthropods. Brachyuran crabs reach their greatest diversity in tropical and temperate regions of the world (Rahman *et al*., 2008)**.** Bangladesh has got diverse freshwater habitat and ecosystem. Crabs are found in almost aquatic ecosystem. Crabs are an important exportable fishery items and hidden resources of Bangladesh (Ahmed, 1991). Food-borne trematodiasis (FBT) is an important group of neglected tropical diseases, which are zoonotic as they are transmitted by the consumption of raw or undercooked aquatic foods that harbour the metacercaria of the flukes affecting a large section of population, particularly in Southeast Asian countries. Parasitic zoonoses are well recognized and important public health problems both in developing and developed countries. The burden of diseases caused by food-borne pathogens (microbes, protozoa and helminths) remains largely unknown. Emerging microbial and protozoan zoonoses are major causes of diarrhoeal disease in humans worldwide (Koopmans and Duizer, 2004; Smith *et al*., 2007). In fact, more than 100 species of food-borne trematodes are known to infect humans, including liver flukes, lung flukes, and intestinal flukes. More than 40 million people infected and more than 10% of the world's population at risk of food borne trematode (FBT) infection (Keiser and Utzinge, 2005, 2009; Sripa *et al*., 2010; Toledo *et al*, 2011). FBTs are endemic in various parts of the world, particularly Southeast Asian regions (Dixon and Flohr, 1997). Fishes and crustaceans may harbour the infective metacercarial stage of a large number of trematodes, which are responsible for FBT (WHO, 1995; Lun *et al*. 2005; Bullard and Overstreet, 2008), thus serving as vector for some human helminthic diseases. The Crustacea-borne trematode infections are caused by fluke parasites belonging to families Paragonimidae, Microphallidae, Lecithodendriidae, Brachylaimidae etc. (Yamaguti, 1971; Anantaraman and Subramoniam, 1976; Janardanan *et al*., 1987). Among these infections, *Paragonimus* spp. causing paragonimiasis pose a continuing public health problem (WHO, 1995; Blair *et*

al., 1999; Nakamura-Uchiyama *et al*., 2002). They occur in a number of countries in several regions of Asia, Africa and Latin America (Toscano *et al*., 1995). Seven *Paragonimus* species, namely *P. heterotremus*, *P. westermani*, *P. skrjabini*, *P. vietnamensis*, *P. proliferus*, *P. bangkokenis* and *P. harinasutai*, have been identified in Vietnam (Doanh *et al*., 2013). Lung flukes, *P. westermani*, *P. miyazakii*, and *P. ohirai* occur in Japan where the first 2 species are zoonotic pathogens that cause pulmonary and sometimes extrapulmonary diseases (Sugiyama *et al*., 2013). There are about 50 species of *Paragonimus* which 11 are known to cause human infections. *Paragonimus westermani* has been regarded as the most common and widely distributed human pathogen in Asia (Miyazaki, 1974). Traditionally, these infections were limited, for the most part, in populations living in low income countries, particularly in Southeast Asia, and were associated with poverty (Toledo *et al*., 2012).

Paragonimus spp. (*P. heterotremus*, *P. westermani*, *P. miyazakii manipurinus*, *P. skrjabini*) has been reported from Manipur, Nagaland, Arunachol, Meghalaya in India (Singh and Singh, 1997., Narain *et al.*, 2003, Singh *et al.*, 2009). Therefore, there is a high chance for their existence in Bangladesh. Hence, the present study was primarily aimed to investigate the occurrence of *paragonimus* spp. in freshwater crab in different areas of Bangladesh.

Along with *paragonimus* metacercariae, *microphallid* metacercearіae has also been found in crabs of Meghalaya, northeast India (Gowsami *et al*., 2013). The family Microphallidae Ward, 1901 indicates small-sized (usually, 1mm) digenean taxa representing more than 160 species under 28 genera arranged in ten subfamilies. They are characteristically found in the intestine of all groups of vertebrates, mainly Charadriiformes birds such as herring gulls, shore-birds, ducks (Martorelli *et al*., 2004) and among mammals, especially rodents (Deblock, 1971). Of these taxa, only *Spelotrema brevicaeca* is reported to infect humans (Fried *et al*., 2004). Infective metacercarial stages of *microphallid* flukes commonly occur in Crustacea (Heard & Overstreet, 1983; Pung *et al*., 2002), intermediate hosts in which they undergo extensive organogenesis (Caveny and Etges, 1971). Many researcher have made significant contributions to the study of *microphallid* life cycles (Cable and Hunninen, 1940; Stunkard, 1957; James, 1968). *Microphallid* trematodes of shore-birds have demonstrated the involvement of various

crustacean and xiphosuran second intermediate hosts, such as crabs, sand crabs, barnacles, and the king crab. There are some species of Microphallus reported in India like *Microphallus nicolli, M. dicaecus* and *M. indicus* (Anantaraman and Subramoniam, 1976).

Freshwater crabs plays an important role to transmit metacercariae into the final host. Crabs contain different types of metacercariae as second intermediate hosts. Poor and marginal people of Bangladesh takes freshwater crabs seasonally and the tribal people takes raw, uncooked or pickled of crabs regularly. The present study was aimed to find out the trematode infections prevailing among the edible crab species, the potential intermediate hosts for digenetic flukes in selected localities of Bangladesh. As the metacercariae is distributed in Northeastern India, its prevalence in Bangladesh is not surprising. Unfortunately, no attempt has been paid on the comprehensive studies of the crab borne trematode infection in Bangladesh. This is quite difficult to track adult parasite collection in human. For this reason, freshwater crabs, the second intermediate host of zoonotic trematodes which carry the metacercariae of these parasites species are tracked to observe the prevalence. We collected crabs from at least one district in every division of Bangladesh. These data will be useful for establishing countermeasures needed to control these fluke infections among the freshwater crabs eaters.

The present work has been taken into consideration to

i. investigate the freshwater crab metacercariae;
ii. identify the metacercariae based on morphology;
iii. outline the geographic distribution of the metacercariae in Bangladesh.

CHAPTER 2

REVIEW OF LITERATURE

Freshwater crabs are found throughout the tropical and sub-tropical regions of the world. They live in a wide range of water bodies, from fast-flowing rivers to swamps, as well as in tree boles or caves. The majority of species are narrow endemics, occurring in only a small geographical area (Hasan and Rashid, 2016). Crabs are a good source of food to different communities of the people as well as play an important role in the food chain of aquatic ecosystem. Now a days, crabs play a significant role in the fishery wealth of many nations (Chhapgar, 1991). *Sartoriana spinigera* is an important edible freshwater crab species. *Sartoriana spinigera* are abundantly found in the mud wetland of Bangladesh, Pakistan and India (Assam, Bihar, Uttar Pradesh, West Bengal). The wetland habitats are the most favourable shelter for the propagation of this crab. These crabs are found crawling, burrowing and even buried in the mud soil of the littoral region of wetlands (Mahapatra *et al*., 2017). *Potamiscus manipuriensis*, also harboured other metacercariae of the microphallid trematode genus *Microphallus*, beside *P. heterotremus*. Metacercarial stages of *Microphallus* have been earlier reported from sand crabs and brackish-water prawns (Anantaraman and Subramoniam, 1976; Jayasree *et al*., 2001).

In Meghalaya, Northeast india, *Barytelphusa* crabs were found to be infected with metacercariae. The prevalence of infection was 91.05% and the intensity was high in crabs collected from Meghalaya in where 285 metacercariae were recovered from a single crab. In Assam, however, the rate and intensity of infection were rather low 15.70% along with a maximum of 23 metacercariae recovered from single host. Metacercariae of *M. indicus* have been reported from *B. lugubris* in Meghalaya (Goswami *et al*., 2013). Significant information on the molecular characterization of microphallid digeneans has become available in recent years (Tkach *et al*., 2000, 2003).

In Manipur, Northeast india, The status of metacercarial infection prevalent in crab hosts (represented by *Barytelphusa lugubris masoniana* (Gecarcinucidae) and *Potamiscus manipuriensis* (Potamidae) from the study area. Both species were found to be infected

with metacercariae where infection rate was 28.90 %. Based on morphological and morphometric criteria, the recovered metacercariae were identified as two species of the genus Paragonimus (family Paragonimidae) and two more types representing the genus *Microphallus* (family Microphallidae). The crab species (*B. lugubris masoniana* and *P. manipuriensis*) were found infected with 4 different types of metacercariae representing the genera *Paragonimus* (Troglotrematidae) and *Microphallus* (Microphallidae). The paragonimids showed a higher rate of occurrence (4–25%) compared to microphallids (15%). The crustaceans surveyed emerged as prospective intermediate hosts for lungflukes (Athokpam and Tandon, 2013).

In Madras, India, A microphallid metacercaria resembling that of *Microphallus nicolli* (*Spelotrema nicolli*). (Baer, 1943) is reported from the sand crabs, *Emerita asiatica* and *Albunea symnista*, on the Madras Coast and tentatively identified as belonging to the genus *Microphallus*. There hosts were observed highest infection, all of 144 (100%) *E. asiatica* crabs (16-31mm long) and 22 of 23 (96%) *A. symnista* (13-27mm long) examined between July and August 1974 having been infected in varying intensities. Strangely, only the female crabs harboured the metacercariae, in the connective tissue of the ovaries but the males being free. The ovary appeared to be the primary seat of infection, though parts of the liver may be involved (Anantaraman and Subramoniam, 1976). Species of *Microphallus* reported from India include *M. dicaecus* and *M. indicus*, of which *M. indicus* has been synonymized with *Bengaliniella dicaecus*.

Apart from *Paragonimus*, many other trematodes, members of the family *Microphallidae* in particular, utilize crabs as the intermediate host (Anantaraman and Subramoniam, 1976; Heard and Overstreet, 1983; Pung *et al*., 2002). The family Microphallidae (Ward, 1901) indicate a large assemblage of small sized (usually < 1mm) digenean taxa which characteristically harbor the intestine of all groups of vertebrates, mainly ducks (Martorelli *et al*., 2004) and mostly rodents among mammals (Deblock, 1971, 2008). As for cases of human infections by microphallids, few such as *Spelotrema brevicaeca*, *Gynaecotyla squatarolae* is reported to infect humans (Fried *et al*., 2004; Chung *et al*., 2011).

In America, The genus *Microphallus* has been reviewed repeatedly but its limits and valid species are still uncertain. It was erected by (Ward, 1901) to contain *Distomum opacum* (Ward, 1894), a parasite of the intestine of the bowfin, *Amia calva*, taken in Lake St. Clair, Michigan. *Microphallus ovatus*, was described by (Osborn, 1919) from cysts in the liver of unidentified crayfish and the intestine of small-mouth black bass, *Micropterus dolomieu*, taken from Lake Chautauqua, New York. (Van Cleave and Mueller, 1934) reported *M. opacus*, from *Ameiurus nebulosus* and *Ambloplites rupestris*, and *M. ovatus* from *Esox niger* and *A. rupestris*, taken in Oneida Lake, New York all specimens of *Microphallus* had been described from fishes. (Rausch, 1946) reported heavy infections by *M. ovatus* in three species of turtles, viz., *Chrysemys belli marginata*, *Chelydra serpentina*, and *Graptemys geographica*. (Rausch, 1946), from a raccoon, *Procyon lotor*, reported specimens that belonged to the genus *Microphallus* but differed from both *M. opacus* and *M. ovatus*.

The first report of an experimentally demonstrated life cycle in the family Microphallidae, that of *Cornucopula nassicola* (Cable and Hunninen, 1938; Rankin, 1939) was made by Rankin (1939) in an abstract published in December. (Deblock and Rose, 1964) described a microphallid species from wild ducks, *Anas platyrhynchos* L., taken along the Somme near Boves, France. (Deblock and Rose, 1966) listed five species of the genus *Microphallus* for which the life-cycles had been experimentally demonstrated, viz., *M. nicolli* (Cable and Hunninen, 1940); *M. pygmaeum* (Levinsen, 1881) by Belopolskaia in 1949; *M. similis* (Jagerskiold, 1900) by Stunkard in 1957; *M. somateriae* by Tchoubryk in 1957 and Kulatschkowa in 1958, as reported by Belopolskaia (1963); and *M. papillorobustus* (Rankin, 1940) by Rebecq in 1964. The metacercarial cysts of *M. limuli* from *L. polyphemus* are slightly larger than those of *M. claviformis* as described by Rebecq (1964). The adults of *M. limuli* are distinctly larger, especially those from birds reported by (Stunkard, 1953). The worms mature at a smaller size in mammals. In *M. claviformis* the suckers are about the same size, whereas in *M. limuli*, the acetabulum is distinctly smaller than the oral sucker. Furthermore, in *M. limuli* the acetabulum and gonads are situated farther posterior in the body.

Chung *et al*., 2011 reoported that Microphallidae is a large family of small flukes found in the intestines of birds, that have consumed crustaceans such as crabs, cray fishes. In South Korea, these 4 species have been described from crustacean hosts: *Microphalloides japonicus* from *Helice tridens tridens*, *Levinseniella* spp. from *Macrophthalmus japonicus*, *Gynaecotyla squatarolae*, and *Microphallus koreana* from *Macrophthalmus dilatatus.* In this study, they discovered the adult worms of *Maritrema obstipum* from the intestine of migratory birds, identified them as the natural definitive hosts of *M. obstipum* in Korea.

CHAPTER 3

MATERIALS AND METHODS

3.1. Sampling area and crabs catching

A total of 261, crustacean the second intermediate hosts, mainly crabs, were collected in three different predominant seasons (Winter, Summer and Rainy) from different geographic area of Bangladesh namely Southeastern hills (Bandarban), Haor and low land (Sylhet, Sunamgonj and Kishorgonj), Barind area (Rajshahi, Rangpur, Naogaon, Thakurgoan and Sirajgonj), the coast (Barguna, Cox'sBazar), the plain Char land (Brahmanbaria, Narsingdi) and the high land (Meherpur, Jashore, Magura and Faridpur). Crab specimens were collected by using cast net, seine net, push net, dip net, and trap from aforesaid areas. After collection, the photographs were taken immediately and the fresh specimens were brought to the laboratory. For detail studies, the collected crabs (fresh and fixed) were shifted to the laboratory of the Department of Microbiology and Parasitology, Sher-e-Bangla Agricultural University, Dhaka. For future studies, the specimens were finally preserved in 70% alcohol in plastic container or kept in the bucket with full of water.

A.

B.

C. **D.**

Figure 1: Crabs trapping and collection from Haor. **A+B.** Pulling of cast net, **C.** Collected crabs in net, **D.** Live crabs in bucket. .

3.2. Crabs identification

The collected crab specimens were measured carefully at the Laboratory, and were identified by using the keys of (Alcock, 1910) and the guidelines and descriptions of Mahapatra *et al*., 2017 and Ahmed *et al*., 2008. For identifications of specimens morphological characteristics were considered from the fresh and preserved crab as well.

A. **B.**

C. **D.**

Figure 2: The dorsal and ventral views of the male and female crab. **A.** Dorsal view of female crab, **B.** Ventral view of female crab, **C.** Dorsal view of male crab, **D.** Ventral view of male crab.

3.3. Procedures of collection and preservation of metacercariae

As the crustacean hosts are poikilothermic animals, they were immobilized by being immersed in chilled water and killed by inserting forceps into the heart of crabs. This facilitates the removal of the carapace and legs from the body by hand without the aid of any other instruments. After removing the carapace, the internal organs and body muscles from each crustacean host were collected and the leg muscles were also removed with tweezers after breaking the shell by using the scissors and forceps. Removed crabs muscle were blended with 10 ml acid-pepsin (1g pepsin and 1 litre of 0.7% hydrochloric acid (HCl); 1:1000) fluids by using the blenders upto proper paste. Artificial gastric juice (acid-pepsin solution) were used to facilitate the digestion of the host tissue. Blended crab tissues were incubated with artificial gastric juice at 37°C for 1 hour with shaking or stirring. The subsequent digest were filtered through a wire sieve, for example, 1mm mesh, allowed to sediment, and washed with several times (at least 3 times) of tap water. After removal of supernatant, the sediment were taken on petridish and observed under a light microscope for collection of metacercariae. Then, the metacercariae were withdrawn by the help of dropper one by one. The metacercariae were preserved in 70% alcohol in vial at 4°C for further morphological identification.

A.

B.

C.

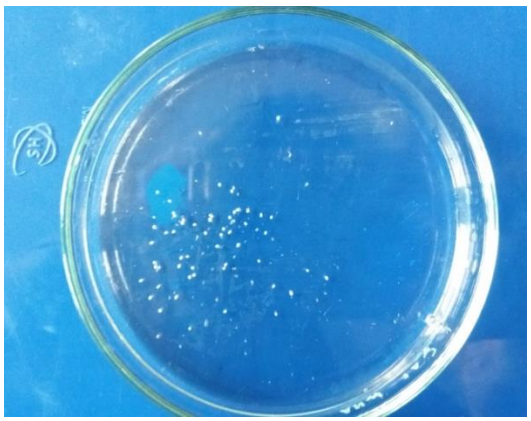
D.

Figure 3: Collection of metacercariae from crabs, **A.** Removal of shell, **B.** Collected muscular parts of crabs, **C.** Muscular parts of crabs in blender, **D.** Blended tissue for proper sedimentation.

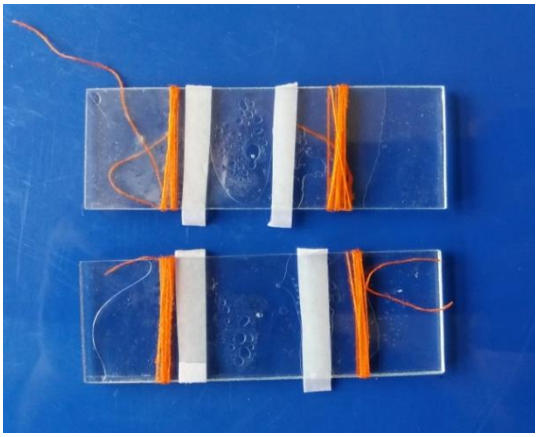
3.4. Whole mounting

Whole mount permanent slide is needed for morphological identification of metacercariae. To process for permanent whole mount, the immediately collected fresh encysted metacercaria were taken on a clean glass slide. Few drops of PBS solution was added and tried to break the outer shell by using fine needle. It needed lots of time to succeed. Then the outer shell membrane were broken and expelled out pyriform shape larva. After that the specimens were made straight with fine brush, put a coverslip and tied it by thread. Then the slide were kept in a couplin jar containing 70% alcohol and kept the specimen at least for overnight and the flattened specimen were processed for whole mount.

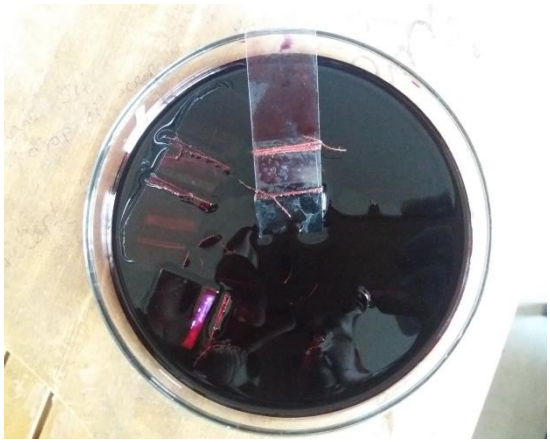
For staining, specimens were transferred to 50% alcohol for and washed in distilled water for an hour each. Then specimens were put in haematoxylin solution for 24 hours and were checked several times to observe the condition of taking stain. The excessive stain was removed by acid alcohol. The stained specimen was dehydrated with ascending grades of alcohol (from 70% to 100%), cleared by xylene and mounted with Canada balsam. Then the mounted slide were dried for further identification.



A.



B.



C. **D.**

E. F.

Figure 4: Excysting of metcercariae and making whole mount, **A.** Encysted metacercariae, **B.** Making flattened by thread, **C.** preserved at 70% **, D.** Staining by haematoxylin, **E.** De-staining through acid alcohol, **F.** Dehydrated by ascending alcohol.

CHAPTER 4

RESULTS AND DISCUSSION

RESULTS

4.1. Identification of crab intermediate host

For identification of freshwater crabs morphological characteristics were studied from the fresh and preserved crab as well. The crabs were brownish yellow in color. The carapace was brown to dark brown in colour while the chillete was orange to brown. The carapace texture of the crab was very much oily; smooth in texture and semi-triangular in shape. A large 'V' shaped patter with dark brown spot were present on the carapace of the crab. It was observed that the female possess a broader abdomen than that of the male (Wood-Mason, 1871). The collected freshwater crabs were identified as *Sartoriana spinigera* following the keys and description of Wood-Masson (1871).

4.2. Morphological observations of *Microphallus indicus* metacercariae

Through examination of 261 freshwater crabs, a large number of Microphalid metacercariae were recovered. The metacercariae were excysted and morphologically identified based on the keys and description of (Mukherjee and Ghosh, 1967) as *Microphallus indicus.*

The metacercarial stage of *M. indicus* is (Family: Microphalidae) is found in second intermediate host different types of freshwater crabs such as, *Barythelphusa lugubris mansonia* and *Potamiscus manipuriensis, Sartoriana spinigera* etc. The adults are characteristically found in the intestine of all vertebrates groups, mainly Charadriiformes birds and among mammals, especially rodents, turtles, raccoons.

A large number of metacercarial cysts (1-63) were recovered from the infected crabs (Figure: 5A). The cyst was elliptical in shape and had a prominent thick wall composed of two layers, where the outer layer being thick but transparent and the inner layer was thin. The vitelline glandular cells were clearly visible in two opposite poles of the encysted metacercariae (Figure: 5B).

A whole mount was prepared after excysting the metacercariae to study the detail morphology under microscope. The body was pyriform in shape and minute in size. The oral sucker was subterminal; the ventral sucker was single, well developed and post-equatorial in position (Figure: 5C). The prepharynx was conspicuous; pharynx was small, short and muscular. The oesophagus was long, bifurcating in mid body region. Intestinal caeca was short, pretesticular, preacetabular (Figure: 5C). Testes were symmetrical, located one on either side of ventral sucker; cirrus (phallus) pouch was present, curved in shape located at the level of caecal bifurcation; cirrus was well developed and conical in the shape. Ovary was located on right side of ventral sucker or slightly overlapping right testis (Figure: 5E). Vitelleria commencing from level of testes just behind the cirrus pouch, extending up to the posterior end of excretory vesicle; vitelline glandular cells were arranged in two groups, one with seven lobes on right, the other with six on left (Figure: 5F). Excretory vesicle was 'V' shaped with the terminal excretory pore (Figure: 5G). These morphological characters were in accordance with those described by Gowsami *et al*., 2013. With these features of morphological features, the precocious larval stage recovered from the crab belonged to family Microphallidae (Ward,1901) and the species *M. indicus.*

The taxonomic status of *M. indicus*

Classification

Kingdom: Animalia

Phylum: Platyhelminthes

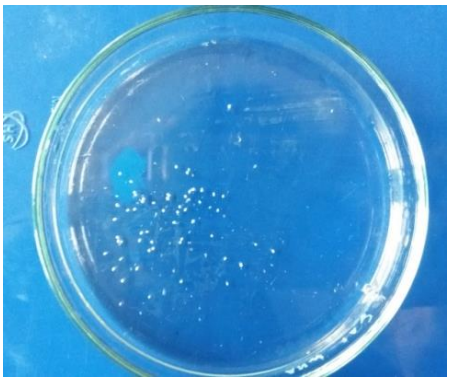
Class: Trematoda

Family: Microphallidae (Ward, 1901);

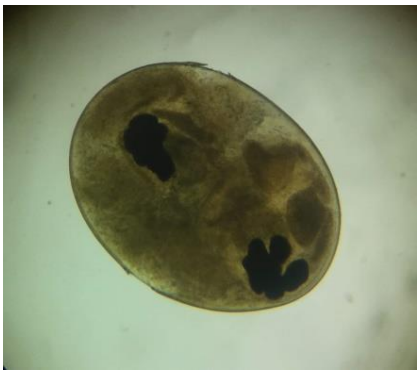
Subfamily: Microphallinae (Ward, 1901);

Genus: *Microphallus* (Ward, 1901);

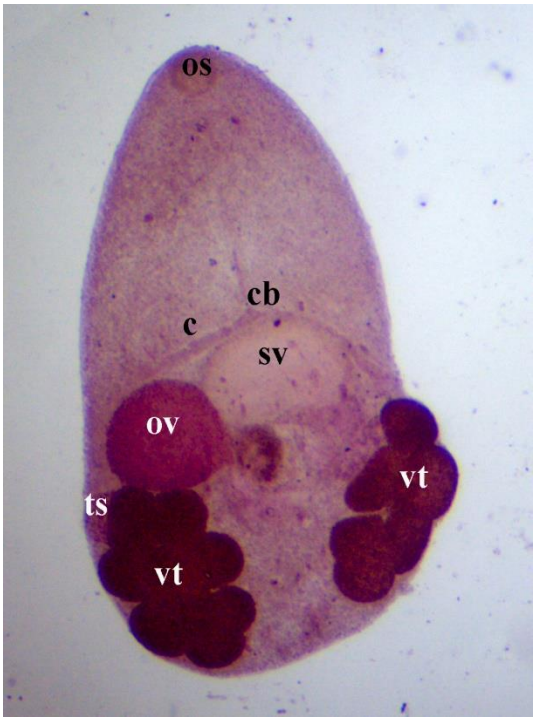
Species: *Microphallus indicus* (Mukherjee & Ghosh, 1967).



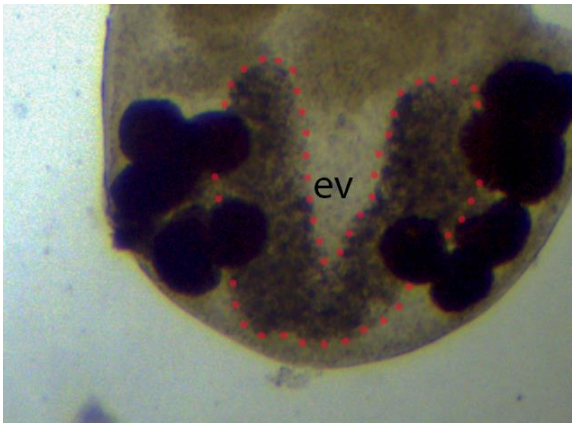
(A)



(B)



(C)



(D)

(os = oral sucker, cb = caecal bifurcation, c = caeca, sv = seminal vesicle, ac = acetabulum, ov = ovary, ts = testes, vt = vitellaria, ev = excretory vesicle)

Figure 5: Different body parts of larval stage of *Microphallus indicus*. **A.** Metacercariae of *M. indicus* from infected crabs. **B**. Encysted Metacercariae preesed with glass slide (4X). **C.** Excysted metacercariae with whole mount (Ventral view). The 'os' indicate the Subterminal, oral sucker. Ovary located on right side of ventral sucker. Vitelline glandular cell in right 8 and in left 7 (10X). **D.** Excretory vesicle 'V' shaped, excretory pore terminal (10X).

4.3. Prevalence

4.3.1. Overall prevalence

The study was carried out in a total of 261 crabs which were collected from different geographic areas of Bangladesh. Discussed among 261 crabs, 66 (26%) crabs were found to be infected with Microphalid metacercariae. Fortunately, no crabs were found to harbor the metacercariae of *Paragonimus* spp. in any of the areas studied.

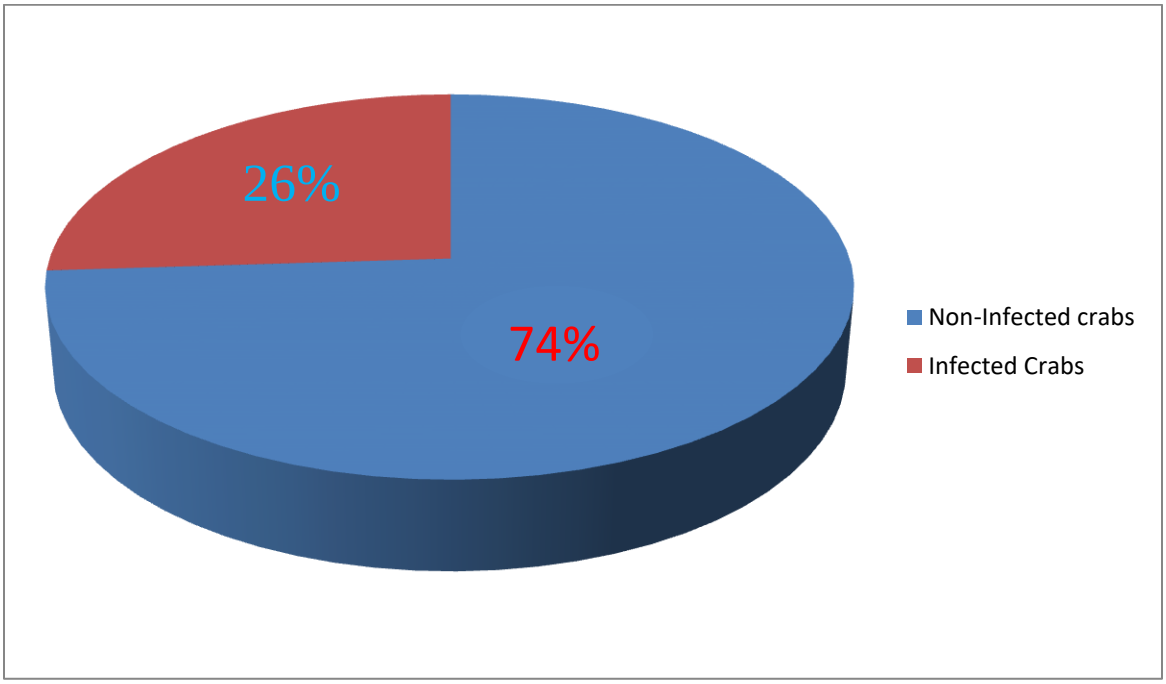


Figure 6: Overall prevalence of metacercarial infection in freshwater crabs**.**

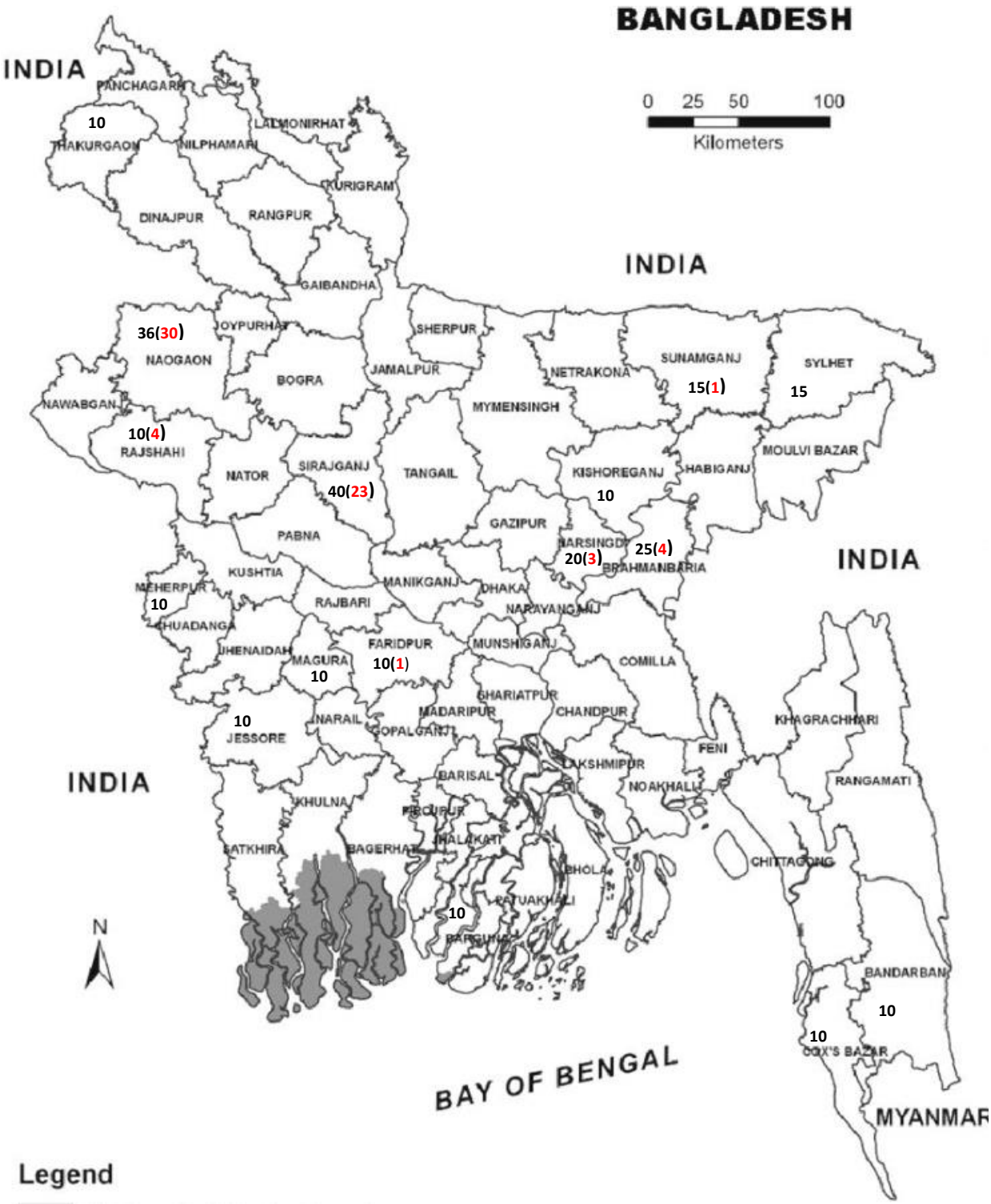


Figure 7: Geographic distribution of *M. indicus*.

4.3.2. Prevalence of metacercariae in freshwater crabs of different Sampling area

The crabs (*Sartoriana spinigera*) were collected from Sirajgonj (n=40), Naogoan (n=36), Rajshahi (n=10), Rangpur (n=10), Thakurgoan (n=10), Jashore (n=10), Faridpur (n=10), Meherpur (n=10), Magura (n=10), Barguna (n=10), Brahmanbaria (n=25), Narsingdi (n=20), Sylhet (n=15), Sunamgonj (n=15), Kishorgonj (n=10), Cox'sBazar (n=10) and Bandarban (n=10). The highest metacercarial infection (83.33%) was observed in the crabs collected from Naogaon, followed by Sirajgonj (57.5%), Rajshahi (40%), Brahmanbaria (16%), Narsingdi (15%), Faridpur (10%), and Sunamgonj (7%). None of the crabs collected from Rangpur, Thakurgoan, Jashore, Meherpur, Magura, Barguna, Sylhet, Kishorgonj, Cox'sBazar and Bandarban was found to be infected with any microphallid metacearia (Table 1).

Table 1: Prevalence and burden of Microphallid metacercariae in freshwater crabs collected from 17 districts in Bangladesh

Location	Coordinate	No. of crabs		Prevalence	No. of mc range (n)	No. of mc per crab (n) / Average	F value
		Examined	Infected				
Sirajgonj	24.136021 89.581322	40	23	57.5%	1-33	4.78	
Naogoan	24.919133 88.749633	36	30	83.33%	1-63	13.43	
Rajshahi	24.642524 88.621746	10	4	40%	1-12	2.0	
Rangpur	25.879722 89.226848	10	0	0%	-	-	
Thakurgoan	26.114020 88.442169	10	0	0%	-	-	
Jashore	23.217547 89.348854	10	0	0%	-	-	
Faridpur	23.395707 89.988734	10	1	10%	0-5	0.5	
Meherpur	23.856022 88.702222	10	0	0%	-	-	*5.70
Magura	23.317698 89.426530	10	0	0%	-	-	
Barguna	22.417415 90.165138	10	0	0%	-	-	
Brahmanbaria	24.031861 90.998189	25	4	16%	2-6	0.6	
Narsingdi	24.207654 90.762637	20	3	15%	4-60	3.75	
Sylhet	24.830141 91.733862	15	0	0%	-	-	
Sunamgonj	25.038088 91.388767	15	1	7%	0-5	0.5	
Kishorgonj	24.416618 90.655241	10	0	0%	-	-	
Cox'sBazar	21.432415 92.100793	10	0	0%	-	-	
Bandarban	21.422969 92.174151	10	0	0%	-	-	
Total		**261**	**66**	**26%**			

N.B. mc = metacercariae, no. = number
* indicates that 1% level of significance

4.3.3. Prevalence of Microphalid metacercariae in freshwater crabs in different water bodies.

The highest number of infected crabs were found the in rivers (56%), followed by haor, canal and ditches (5%) and ponds (0%) (Table 2).

Table 2: Prevalence and burden of metacercariae in freshwater crabs according to types of waterbodies.

Location	No of crabs		Prevalence	No of mc range
	Examined	Infected		
River	121	61	56%	1-63
Haor, canal and ditches	100	5	5%	1-33
Ponds	40	0	0%	-

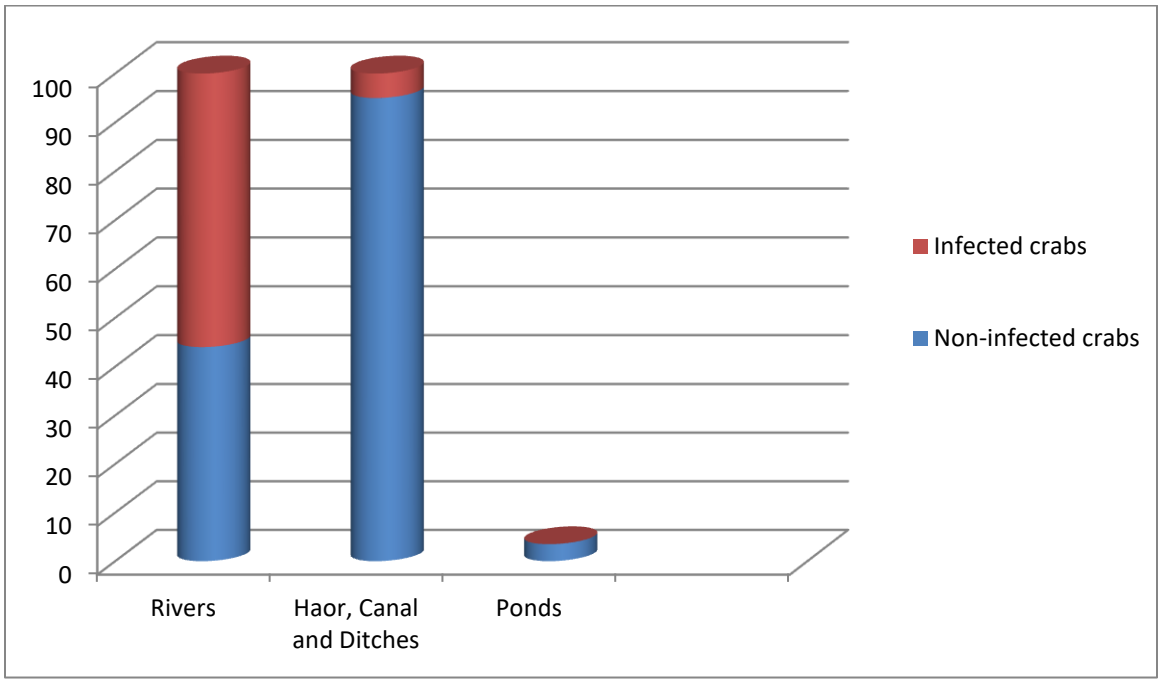


Figure 8: Chart shows the prevalence of according to water bodies.

DISCUSSION

In the present study, all the trapped crabs were *S. spinigera* species. This study gives an overview on the metacercarial infection of freshwater crabs in Bangladesh. The prime objectives of this study was to investigate the existence of *Paragonimus* metacercariae in freshwater crabs. But none of the examined crabs was found to harbor *Paragonimus* metacercariae. In this study, *M. indicus*, is going to be reported for the first time from Bangladesh.

Microphallus indicus were found to be distributed in freshwater bodies of different district in Bangladesh. The occurrence of *M. indicus* was reported from Northeast India namely, Assam, Meghalaya, Manipur (Athokpam and Tandon., 2013; Goswami *et al*., 2013) and *M. nicolli* from South (Madras) India (Anantaraman and Subramoniam, 1976). Therefore, the existence of this metacercariae from freshwater crabs in Bangladesh is not surprising.

There are six genera under the tribe; these are *Microphallus* (Ward, 1901); *Megalophallus* (Cable *et al*., 1960); *Atriophallophorus* (Deblock and Rose, 1964); *Megalophalloides* (Ching and Ibanez, 1976); *Megistospermaticus* (Deblock and Canaris, 1999); and *Rhyncostophallus* (Deblock and Canaris, 1997). Through detail morphological studies, several body points were found useful for the identification of the metaceareariae. The metacercariae had linguiform, flattened body with sub-terminal oral sucker. The ventral is sucker post equatorial. The oesophagus is medium sized; the caecum is short and divided into two at the level of ventral sucker. The testes are ovoid with post ovarian. The ovary is directly in front of right testes. The vitellaria has two relatively large clusters, each comprising 7-8 follicles. The excretory vesicle is V-shaped. Considering all the morphological characters and following the keys and description of Yamaguti, 1971 and Deblock, 2008, the metacercariae were identified as *M. indicus. Microphallus indicus* was differentiated from *M. nicolli* by the morphological features like Y-shaped excretory pore in the later species where larva is enclosed with 3 layer spherical cyst (Anantaraman and Subramoniam, 1976).

In addition to these, microphallid metacercarial stages have also been reported from sand crabs and brackish-water prawns near the south-eastern coast of the Indian subcontinent

(Anantaraman & Subramoniam, 1976; Jayasree *et al*., 2001). A comparison of morphological features of various microphallid species described so far from crustacean hosts in India reveals a close similarity to the present metacercarial stage, with *M. indicus* (Mukherjee and Ghosh, 1967), originally described from a reptilian final host. Microphallids are known for rapidly attaining sexual maturity (Ching, 1963), and therefore, the metacercarial stage and the adult form are quite similar morphologically.

The present study showed that *S. spinigera* harboured the microphalid metacercaria in different districts of Bangladesh. Goswami *et al*., (2013) reported that Metacercariae of *M. indicus* from *B. lugubris* in Meghalaya, Assam of northeast India. *Potamiscus manipuriensis* crabs also reported to harbour the microphallid trematode *Microphallus* along with *Paragonimus heterotremus*. Anantaraman and Subramoniam, (1976) and Jayasree *et al*. (2001) reported that metacercarial stages of *Microphallus* had been earlier recovered from sand crabs and brackish-water prawns in sea coast of Madras, India. *Sartoriana spinigera* are abundantly found in the mud soil wetland of Bangladesh, Pakistan and India (Assam, Meghalaya, Manipur, Arunachal, Bihar, Uttar Pradesh, West Bengal). Therefore, they can act as second intermediate host of the microphallids in Bangladesh.

In the present study, 26% crabs were found to be infected with *M. indicus* metacercariae with the highest infection (83.33%) in Naogaon distrcict, Northern parts of Bangladesh. Highest number of metacercariae (63) were collected from a single crabs. Goswami *et al*., (2013) showed that the prevalence of metacercarial infection was 91.05% and the intensity was high in crabs collected from Meghalaya and up to 285 metacercariae were recovered from a single crab. In Assam, however, the prevalence of infection were 15.70% with a maximum of 23 metacercariae recovered from singe crab host. Athokpam and Tandon, (2013) reported that 15% crabs are infected with Microphallid species in Manipur, India. Anantaraman and Subramoniam, (1976) reported that 100% infection were in *Emerita asiatica* crabs and 96% infection were in *Albunea symnista* crabs of Madras coast with *M. nicolli.*

During this study, it was revealed out that highest infection were found in Naogaon district (83.33%) followed by Sirajgonj (57.5%), Rajshahi (40%), Brahmanbaria (16%), Narsingdi (15%), Faridpur (10%), and Sunamgonj (7%). None of the crabs found to be infected in Rangpur, Thakurgoan, Jashore, Meherpur, Magura, Barguna, Sylhet, Kishorgonj, Cox'sBazar and Bandarban. Northern parts of Bangladesh showed highest metacercarial infection due physiological and pathological causes of crabs. Availability and intensity of intermediate crabs host and final host could be the main reason for the prevalence of *M. indicus.* Northern parts of Bangladesh mainly river based where the water is available all the year round. So, the crabs and snails inhabit in these area whole year. AEZ-3, AEZ-4 and AEZ-5 containing northern parts of Bangladesh namely Naogaon, Rajshahi, Natore, Pabna, Sirajgonj that conveys common rivers (Dharla, Dudhkumar, Tista, little Jamuna, Atrai, Brahmaputra) and beels (Chalanbeel). This may be the vital source for this metacercarial infections. A huge amount of wild ducks live in these area which transmitted eggs to intermediate host via feces.

The present study showed that the highest prevalence was observed in the crabs trapped from rivers (56%), followed by haor, canals and ditches (5%) and ponds (0%). Most of the river of Bangladesh originates from Assam, Meghalaya, Manipur, Sikkim of the neighbouring country, India. There are also some common rivers between Bangladesh and India. So, the intermediate host snails, cray fishes and crabs can share the habitats in both the countries. Due to availability of food and water all over the year, crabs get good environment in the river where they can complete their life cycle easily. Due to geographical location of Bangladesh, the water of rivers are decreased during winter season. Thus, the intermediate host, crabs are predated by the final host like shore birds, herring gulls, jackals, wild ducks, turtle etc. In the final host, the life cycle of *M. indicus* is completed. Moreover, migratory water fowl migrates in Bangladesh during winter season which can play an important role in dispersing parasites. Adults of *Maritrema obstipum* (Digenea: Microphallidae) were found in the intestines of migratory birds, including the Kentish plover (*Charadrius alexandrines*), sanderling (*Crocethia alba*), Mongolian plover (*Charadrius mongolus*), and red-necked stint (*Calidris ruficollis*) collected from Korea which were migratory birds. (Chung *et al*., 2011).

Again, the crabs in the haor, canal and ditches migrates in river during winter season due to decreased water level; and thereby, the infection in the river may increase. Due to the lack of final host scavenging at pond and no connection with infected source, there are no infection in the ponds. For this reason, the present study showed that river crabs are mostly infected by *M. indicus* metacercariae than haor, canals, ditches and ponds.

CHAPTER 5

SUMMARY AND CONCLUSION

This study was carried out in the 17 districts of Bangladesh with an aim to find out the prevalence and morphological identification of metacercariae found in freshwater crabs of rivers, haors, canals, ditches and ponds. A total of 261 crabs were captured by using seine nets, cast nets and sometimes through bamboo traps. Overall 26% crabs were found to be infected by *M. indicus*. The results showed that the highest metacercarial infection (83.33%) was observed in Naogaon, followed by Sirajgonj (57.5%), Rajshahi (40%), Brahmanbaria (16%), Narsingdi (15%), Faridpur (10%), and Sunamgonj (7%). The results showed that highest intensity of metacercariae in a single crab was 1-63 with an average of 13.43. The metacercarial infection were nil in Rangpur, Thakurgoan, Jashore, Meherpur, Magura, Barguna, Sylhet, Kishorgonj, Cox'sBazar and Bandarban districts. The results revealed that the highest number of infected crabs were found the in rivers (56%) where haors, canals and ditches were observed (5%). But in ponds, none of the crabs were found to be infected with Microphallid metacercaria. *Microphallus indicus* is going to be reported for the first time from Bangladesh.

Bangladesh is a land of rivers, canals, streams, ponds, ditches, and others water bodies. This water are contains a lots of freshwater crabs. The marginal people specially the fishermen and crab eaters having the chance of getting this infection because crabs play an important as second intermediate host of *Microphallus* metacercariae. Further including more samples should be carried out specially those where the tribal people live and eat fresh crabs or uncooked crabs.

REFERENCES

Ahmed, M. K. (1991). A potential aqua-resource of Bangladesh. *Bay of Bengal Program/REP/51*. 95-103.

Alcock, A. (1910). Brachyura I. Fasc. II. The Indian Freshwater Crabs-Potamonidae. *Catalogue of the Indian Decapod Crustacea in the Collection of the Indian Museum.* 135.

Anantaraman, S. and Subramoniam, T. (1976). On a microphallid metacercaria occurring in the ovaries of the sand crabs *Emerita asiatica* and *Albunea symnista* on the Madras coast. *Proceedings of Indian Academy Science B*. **84** (5):192–199.

Athokpam, V. D. and Tandon, V. (2013). A survey of metacercarial infections in commonly edible fish and crab hosts prevailing in Manipur, Northeast India. *Journal of Parasitic Diseases*. **39**: 429–440.

Bharadwaj, O. N. (1962). On the trematode genus *Isoparorchis* Southwell, 1913. *Proceedings of National Academy of Sciences India.* **31**: 105–106.

Bharadwaj, O. N. (1963). The morphology of a new trematode *Mehraformes jabalpurensis* n.g., n. sp. (*Microphallidae*). *Proceedings of National Academy of Sciences India.* **33**: 245–250.

Blair, D., Xu, Z.B. and Agatsuma, T. (1999). Paragonimiasis and the genus *Paragonimus. Advances in Parasitology*. **42**:174–178.

Bullard, S. A. and Overstreet, R. M. (2008). Digeneans as enemies of fishes. *Fish diseases. Science, US.* **2**: 817–976.

Cable, R. M. and Hunninen, A. V. (1940). Studies on the life history of *Spelotrema nicolli* (Trematoda: Microphallidae) with the description of a new microphallid cercaria. *The Biological Bulletin*. **78** (1): 136-157.

Cable, R. M., Connor, R. S. and Balling, J. W. (1960). Digenetic trematodes of Puerto Rican shore birds. *Scientific Survey of Porto Rico and the Virgin Islands*. **17**: 187–225.

Chhapgar, B. F. (1991). Seashore life of India. Nuture Guides, Oxford University Press, Bombay, India. **3**: 18-24.

Caveny, B. A. and Etges, F. J. (1971). Life history studies of *Microphallus opaqus* (Trematoda: *Microphallidae*). *The Journal of Parasitology*. **57** (6): 1215-1221.

Ching, H. L. (1963). The description and life-cycle of *Maritrema laricola* sp. (Trematoda *Microphallidae*). *Canadian Journal of Zoology*. **41** (5): 881–888.

Chung, O. S., Sohn, W. M., Chai, J. Y., Seo, M. and Lee, H. J. (2011). Discovery of *Maritrema obstipum* (Digenea: Microphallidae) from migratory birds in Korea. *The Korean journal of parasitology*. **49** (4): 457-460.

Deblock, S. and Rose, F. (1965). Contribution à l'étude des Microphallidae Travassos 1920 (Trematoda) des oiseaux de France XI-Identification de la cercaire de *Microphallus claviformis* (Brandes 1888). *Bulletin de la Société zoologique de France.* **90**: 299-314.

Deblock, S and Canaris, A.G. (1999). Megistospermaticus kenyensis n. comb. pour *Microphallus kenyensis Canaris*, 1971 (Digenea: Microphallidae)**.** *Systematic Parasitology***. 43:** 65–66.

Deblock, S., Capron, A. And Rose, F. (1961). Contribution a l'etude des Microphallidae Travassos, 1920 (Trematoda). Le genre *Maritrema Nicoll*, 1907: cycle evolutif de *M. subdolum* Jaegerskioeld, 1909. *Parassitologia.* **3**: 105-119.

Dixon, B. R. and Flohr, R. B. (1997). Fish and shellfish-borne trematode infections in Canada. *Southeast Asian Journal of Tropical Medicine and Public Health.* **28**:58–64.

Doanh, P. N., Horii, Y. and Nawa, Y. (2013). *Paragonimus* and Paragonimiasis in Vietnam: an Update. *The Korean Journal of Parasitol*ogy. **51** (6): 621–627.

Fried, B., Graczyk, T. K. and Tamang, L. (2004). Food-borne intestinal trematodiases in humans. *Parasitology Research*. **93** (2): 159–170.

Goswami, L. M., Prasad, P. K., Biswal, D. K., Chatterjee, A. and Tandon, V. (2013). Crustacean-borne infections with microphallid metacercariae (Digenea:

Microphallidae) from focal areas in Meghalaya, north-east India. *Journal of Helminthology*. **87** (2): 222–229.

Hasan, R. and Rashid, H. O. (2016). A Study and Availability Assessment of Freshwater Crabs in the Hill Streams of Bangladesh. *World Applied Science Journal*. **34** (6): 770-775.

Heard, R. W. and Overstreet, R. M. (1983). Taxonomy and life histories of two North American species of Carneophallus (1⁄4Microphallus) (Digenea: Microphallidae). *Proceedings of the Helminthological Society Washington.* **50**: 170–174.

James, B. L. (1968). Studies on the life cycle of *Microphallus pygmaeus* Levinsen, 1881 (Trematoda: *Microphallidae*). *Journal of Natural History*. **2**: 155 – 172.

Janardanan, K. P., Ramanandan, S. K. and Usha, N. V. (1987). On the progenetic metacercaria of Pleurogenoides ovatus Rao, 1977 Trematoda Pleurogenitinae from the freshwater crab, Paratelphusa hydrodromous Herbst, with observations on its in vitro excystment. *Zoologischer Anzeiger*. **219** (5–6): 313–320.

Jayasree, L., Janakiram, P. and Madhavi, R. (2001). Epibionts and parasites of *Macrobrachium rosenbergii* and *Metapenaeus dobsoni* from Gosthani estuary. *Journal of Natural History*. **35** (2):157–167.

Keiser, J. and Utzinger, J. (2005). Emerging foodborne trematodiasis. *Emerging Infectious Diseases.* **11**:1507–1514.

Keiser, J. and Utzinger, J. (2009). Foodborne trematodiases. *Clinical Microbiology*. **22**: 466-483.

Koopmans, M. and Duizer, E. (2004). Foodborne viruses: an emerging problem. *International journal of food microbiology*. **90** (1): 23-41.

Lal, M. B. (1936). A new species of genus *Levinseniella* from the jack snipe, *Gallinago gallinule*. *Proceedings of the Indian Academy of Sciences- section B.* **4** (2): 92–96.

Lun, Z. R., Gasser, R. B., Lai, D. H., Li, A. X., Zhu, X.Q., Yu, X. B. and Fang, Y.Y. (2005). Clonorchiasis: a key foodborne zoonosis in China. *The Lancet Infectious Diseases*. **5** (1):31–41.

Mahapatra, B. K., Pradhan, A. and Bhattacharya, S. (2017). Biology of Tele Kankra, *Sartoriana spinige*ra (Wood-Mason, 1871) from East Kolkata Wetland, India. *International Journal of Fisheries and Aquatic Studies*. **5** (4): 382-387.

Miyazaki, I. (1974). Symposium on Epidemiology of Parasitic Disease. International Medical Foundation of Japan. Lung flukes in the world: Morphology and life history; pp. 101–34.

Mukherjee, R. P. and Ghosh, R. K. (1967). On two new trematodes of the genus *Microphallus*. *Zoologischer Anzeiger*. **178** (5/6): 342–347.

Murhar, B. M. (1960). On a new host record of the trematode *Basantisia ramai* Pande from the pigeon *Columba domestica* Gmel at Nagpur. *Bulletin of the Zoological Society*. **3**: 79–81.

Nakamura-Uchiyama, F., Mukae, H. and Nawa, Y. (2002). *Paragonimiasis*: a Japanese perspective. *Clinics in Chest Medicine*. **23** (2): 409–420.

Narain, K., Devi, K. R. and Mahanta, J. (2003). Paragonimus and paragonimiasis–a new focus in Arunachal Pradesh, India. *Current Science.* **84** (8): 985-987.

Osborn, H. L. (1919). Observations on *Microphallus ovatus* sp. nov., from the crayfish and black bass of Chautauqua, New York. *Journal of Parasitology*. **5** (3): 123-127.

Pande, B. P. (1938). On a new genus of the *Pleurogenetinae* (*Lecithodendriidae*) from a kingfisher. *Annals and Magazine of Natural History*. **2** (8): 199–203.

Pung, O. J., Khan, R. N., Vives, S. P. and Walker, C. B. (2002). Prevalence, geographic distribution and fitness effects of *Microphallus turgidus* (Trematoda: Microphallidae) in grass shrimp (Palaemonetes spp.) from coastal Georgia. *Journal of Parasitology*. **88** (1): 89–92.

Rahman M. A., Rahman M. M., Ahmed A. T. A., Mollah A. R. and Hossain M. A. (2008). A Survey on the Diversity of Freshwater Crabs in Some Wetland Ecosystems of Bangladesh. *International Journal of Sustainable Crop Production (IJSCP).* **3** (3):10-17.

Rankin, J. S. (1939). Studies on the trematode family Microphallidae Travassos, 1921. I. The genus Levinseniella Stiles and Hassall, 1901 and description of a new genus, Cornucopula. *Transactions of the American Microscopical Society*. **58**: 431-447.

Rao, B. V. (1965). Helminth parasites of an Indian jackel (*Canis aureus naria*): *Ancylostoma braziliense* (Gomez 1910) Leiper 1915, *Rectularia affinis* (Jagerskiold, 1904) (Nematoda); and *Spelotrema narii* n. sp. (Trematoda). *Indian Journal of Helminthology*. **17**: 68–84.

Rao, R. (1969). On *Megalatriotrema hispidum*, a new genus and new species of microphallid trematode from frogs in Andhra Pradesh, India. *Annals in Parasitology*. **44** (2): 125–130.

Rausch, R. (1946). New host records for *Microphallus ovatus* Osborn, 1919. *Journal of Parasitology*. **32** (1): 93-94.

Rausch, R. (1946). The raccoon, a new host for *Microphallus* sp. with additional notes on *M. ovatus* from turtles. *Journal of Parasitology*. **32** (2): 208-209.

Rebecq, J. (1964). Recherches systematiques, biologiques et ecologiques sur les formes larvaires de quelques Trematodes de Camargue. Theses presentees a la Faculte des Sciences de l'Universite d'Aix-Marseille. P: 223.

Singh, T. S. and Singh, K. I. (1997). Three Types of Paragonimus Metacercariae Isolated from Potamiscus Manipurensis, in Manipur. *Indian Journal of Medical Microbiology*. **15**: 159-162.

Singh, T. S., Sugiyama, H., Umehara, A., Hiese, S., and Khalo, K. (2009). Paragonimus heterotremus infection in Nagaland: a new focus of paragonimiasis in India. *Indian Journal of Medical Microbiology*. **27** (2): 123.

Smith, H. V., Caccio, S. M., Cook, N., Nichols, R. A. B. and Tait, A. (2007). *Cryptosporidium* and *Giardia* as foodborne zoonoses. *Veterinary Parasitology*. **149** (1-2): 29-40.

Sripa, B., Kaewkes, S., Intapan, P. M., Maleewong, W. and Brindley, P.J. (2010). Food-borne trematodiases in Southeast Asia: epidemiology, pathology, clinical manifestation and control. *Advances in parasitology*. **72:** 305–350.

Stunkard, H. W. (1957). Natural hosts of *Microphallus limuli* Stunkard, 1951. *Journal of Parasitoogyl*. **39**: 2.

Stunkard, H. W. (1957). The morphology and life history of the digenetic trematode, *Microphallus similis*. *The Biological Bulletin*. **112**: 254 – 266.

Stunkard, H. W. (1958). The morphology and life history of *Levinseniella minuta* (Trematoda: *Microphallidae*). *Journal of Parasitology*. **44**: 225 – 230.

Sugiyama, H., Shibata. K., Morishima, Y., Muto, M., Yamasaki, H. and Kawakami, Y. (2013). Current Status of Lung Fluke Metacercarial Infection in Freshwater Crabs in the Kawane Area of Shizuoka Prefecture, Japan. *Journal of Veterinary Medical Science.* **75** (3): 249–253.

Tkach, V. V. and Snyder, S. D. (2007). *Aptorchis megacetabulus* n. sp. (Platyhelminthes: Digenea) from the northern long-necked turtle, *Chelodina rugosa* (Pleurodira: Chelidae), in Australia. *Journal of Parasitology*. **93**: 404–408.

Tkach, V. V., Pawlowski, J. and Mariaux, J. (2000). Phylogenetic analysis of the suborder Plagiorchiata (Platyhelminthes, Digenea) based on partial lsr DNA sequences, Digenea) based on partial lsr DNA sequences. *International Journal of Parasitology*. **30** (1): 83–93.

Tkach, V. V., Littlewood, D. T. J., Olson, P. D., Kinsella, M. J. and Swiderski, Z. (2003). Molecular phylogenetic analysis of the Microphalloidea Ward, 1901 (Trematoda: Digenea). *Systematic Parasitology*. **56**: 1 – 15.

Toledo, R., Bernal, M. D. and Marcilla, A. (2011). Proteomics of foodborne trematodes. *Journal of Proteomics.* **74** (9): 1485–1503.

Toledo, R., Esteban, J. G. and Fried, B. (2012). Current status of food-borne trematode infections. *European journal of clinical microbiology & infectious diseases*. **31** (8): 1705–1718.

Toscano, C., Yu, S. H., Nunn, P. and Mott, K. E. (1995). Paragonimiasis and tuberculosis, diagnostic—a review of the literature. *Tropical Diseases Bulletin*. **92** : R1–R26.

Van Cleave, H. J. and and Mueller, J. F. (1934). Parasites of Oneida Lake fishes. Part. III. A biological and ecological survey of the worm parasities. Bull. N.Y. State College of Forestry. *Roosevelt Wild Life Annals*. **3**: 161-334.

Ward, H. B. (1894). On the parasites of Lake fish. I. Notes on the structure and life history of *Distomum opacum* n. sp. *Proceedings of the American Microscopical Society*. **15** (3): 173-182.

Ward, H. B. (1901). Notes on the parasites of lake fish III. On the structure of the copulatory organs in Microphallus. *Transactions of the American Microscopical Society*. **22**: 175–187.

World Health Organization. (1995). Control of food borne trematode infections. *WHO technical report series*. **849**: 1–157

World Health Organization. (2009). Foodborne trematode infections and taeniasis / cysticercosis. Report of the WHO expert consultation, Vientiane, Lao PDR. 1–50.

Yamaguti, S. (1971). Synopsis of digenetic trematodes of vertebrates. Vols I and II. *Synopsis of digenetic trematodes of vertebrates. Vols I and II.*