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Prevalence of fish-borne zoonotic parasites and their molecular identification in Bhola district of Bangladesh

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Abstract. Fish-borne zoonotic parasites are a rapidly increasing health hazards for human in all over the world. There is a scarcity of information on the fish-borne zoonotic parasite infection in Bangladesh. The present study was aimed to investigate fish-borne helminth parasites in Bhola district. A total of 300 fresh fish samples were collected randomly from different local markets of Bhola district and transported to the laboratory of the Department of Parasitology, BAU. Fishes were homogenized and digested with pepsin-HCl solution for overnight and washed with NaCl solution for 8-9 times and then sediment was examined under a microscope to detect the presence of metacercariae/metacestode. Fishes were found to be infected with multiple infective stages of helminth parasites such as *Opisthorchis* sp., *Clonorchis* sp., *Diphyllobothrium* sp., *Metagonimus* sp. and *Metorchis* sp. In this study, 58% overall prevalence of fish-borne zoonotic parasites was recorded. About 80% metacercariae was found in Loitta (*Harpadon nehereus*) followed by 65% in Spotted snakehead *(Channa punctata)*, 60% in Tilapia *(Oreochromis nilolticus)*, 45% in Stinging catfish (*Heteropneustes fossilis*) and 24.28% in Indian carplet (*Amblypharyngodon microlepis*). The plerocercoid of *Diphyllobothrium* sp. were identified morphologically and was confirmed molecularly by PCR. This study indicates that fish-borne helminth parasites may pose a significant public health risk to the people of Bhola district.

Keywords: Zoonotic Parasites, Fishes, Bhola district.

Introduction

The fish-borne zoonotic parasites (FBZP) affect the human and animal health around the world, particularly in the Asian countries (Sohn 2009). Globally, it is estimated that more than 40 million people have been infected annually with FBZP and approximately 600 million people are at risk of fish-borne zoonotic parasitic infections (Chai *et al.* 2005, WHO 2002; Tantrawatpan *et al.* 2014). Humans are mostly infected with FBZP when they have a traditional custom to consume live, raw, smoked or inadequately cooked fish containing the infective stages, metacercariae and/or metacestodes (Eiras *et al.* 2016). These parasites provoke a remarkable morbidity and cause serious damage to aquaculture, which is a valuable source of food and employment in developing countries. Freshwater and brackish water fish play an important role as the source of human infections with food-borne parasites. (Chai *et al.* 2005, Chai *et al.* 2007, WHO 2002). FBZP have a complex life cycle, where two intermediate hosts are involved. There is a series of developmental stages occur in the freshwater snails (1st intermediate host) where the cercariae are produced. These cercariae emerge from the snails into water and encysted in the fins, skin and muscles of the freshwater fishes especially the cyprinoid fishes (2nd intermediate host) and develop to infective stage, the metacercariae (MC). These metacercariae are responsible for the infection to humans who ingest the raw or undercooked and smoked fish (Sripa *et al.* 2011).

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Recently, these FBZP are becoming an alarming issue for human health throughout the world. The infection of helminths is usually caused by liver fluke, *Opisthorchis viverrini, O. felineus* and *Clonorchis sinensis* which have a major public health threat in the Southeast and East Asian countries (Zheng *et al.* 2017). *O. viverrini* is endemic in Southeast Asian countries, including Thailand, Laos, Vietnam and Cambodia (Sripa et al. 2011). On the other hand, clonorchiasis is predominantly endemic in East Asia, but it may also occur in other regions where there are immigrants from endemic areas (WHO 1995). About 45 million people are infected with opisthorchiasis, and 15 million people are infected with clonorchiasis in East Asia (Keiser and Utzinger 2009, Qian *et al.* 2016). More than 750 million people are at risk to fish borne liver flukes throughout Asia (Nguyen et al. 2013) and 40–50 million people are a□ected with one or more species of fish-borne intestinal flukes and approximately half a billion individuals are at risk globally (Hung *et al.* 2015). Due to proper awareness, lack of information on geographical distribution and disease burden, their public health impact has been neglected for a long time. These parasites live in the bile ducts, gallbladder and liver parenchyma and cause various liver and biliary diseases such as biliary tract obstruction, icterus, chronic inflammation and cholangiocarcinoma (CCA) (Qian *et al.* 2012, Silakit *et al.* 2015, Parkin *et al.* 1993). Recently, the CCA is considered the most severe complication of liver fluke infection and *C. sinensis* and *O. viverrini* infections are classified as "carcinogenic to humans" (Group 1) by the International Agency for Research on Cancer (IARC) (Bouvard *et al.* 2009). Diphyllobothriasis (*Diphyllobothrium latum*) is another an important zoonotic disease that causes epigastric pain, anorexia, abdominal cramps, diarrhea, weight loss and megaloblastic anemia or pernicious anemia in human (Dorny *et. al.* 2009). *Dibothriocephalus latus* and *Gnathostoma spinigerum* have been reported from the fresh water fishes of Bangladesh (Chandra KJ, 2005). Very recently, the fish-borne trematode infections have been reported from wild fishes in Mymensingh and Kishoreganj district of Bangladesh (Labony *et al.* 2020).

The southern coastal rivers of Bangladesh are categorized with high levels of commercial fish harvest which has direct impact on the national economy of the country (Islam, 2003; Sharker et al. 2015). Bhola district is one of the largest islands located in the southern part of the country and considered the hotspot of varieties of fresh and marine water fishes and recognized as the fish landing center. It is assumed that these fishes may harbor the infective stages of fish-borne zoonotic parasites which might pose a great risk to human health. Despite being of great importance, there is a very scarcity of information regarding the fish-borne zoonotic parasites in Bhola district as well as in Bangladesh and the fish-borne zoonotic parasitic infections has yet not detected from this unique southern coastal island of Bangladesh. Therefore, the present study was undertaken to identify the infective stages (metacercariae/metacestode) of fish-borne zoonotic parasites and to explore the prevalence of these parasites from freshwater fishes marketed in Bhola district of Bangladesh.

Materials and Methods

Study area: Sampling of the present study was performed in Bhola district (22°41′15″ N and 90°38′38″ E) (Fig. 1). A total of 15 different local fish markets such as Lalmohan Kacha Bazar, Elisha Ghat, Veduriya Ghat, Bhola Fish Market, Charfassion Bazar and Betua Ghat were selected randomly from Bhola district since it is a coastal area rich in natural water bodies and considered a hotspot of varieties of fresh and marine water fishes.

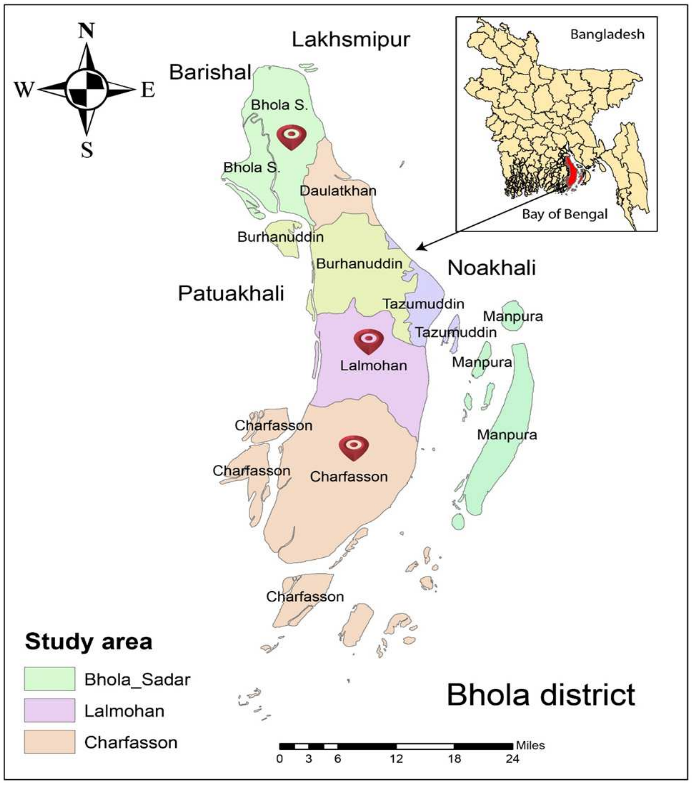

Fig. 1. Map showing the study areas and indicating the most enriched zone of fresh and saline water fishes.

***Sampling of fishes and study period*:** A varieties of fishes were collected randomly from different local markets in the study areas from July 2019 to September 2020. A total of 300 (9.7 kgs) fresh fish samples belongs to ten types, namely, Loitta (Bombay duck) (*Harpadon nehereus*), Gangetic Koi (*Anabas cobojius*), Spotted snakehead (*Channa punctata*), Stinging cat fish (*Heteropneustes fossilis*), Tilapia (*Oreochromis nilolticus*), Indian carplet (*Amblypharyngodon microlepis*), Grass carp (*Ctenopharyngodon idella*), Silver carp (*Hypophthalmichthys molitrix*), Rohu (*Labeo rohita*) and Catla (*Catla catla*) were purchased from local fish markets (Fig. 2) and identified as described by Rahman (2005). After collection, fishes were preserved in ice container and transported to the laboratory of the Department of Parasitology, BAU, Mymensingh and investigated for the presence of the infective stages of helminth parasites associated zoonoses.

***Processing and preparation of fish samples*:** Different species of fishes were weighed and subjected immediately for the detection of metacercariae (MC)/metacestodes or kept separately in polythene bags at −20 ºC until examined within 1-2 week. Fishes were chopped into small pieces consisting of 150–200 g of fishes. The visceral organs of each fish were removed carefully. These pieces were then blended in a blender in which artificial gastric juice containing 0.25% pepsin (LOBA Chemie Pvt. Ltd., Mumbai, India) and 1.5% conc. HCl (Merck, Germany) were added.

***Recovery of the infective stages (metacercariae /metacestode)*:** Processed fish samples were digested with pepsin according to the procedures described previously by Sohn (2009) with a small modifications. In brief, the processed samples were incubated at room temperature overnight in artificial gastric juice under vigorous magnetic stirring conditions. The next day morning, larger particles (scales, fins, bones and undigested materials) were removed by filtering through a sieve (1×1 mm mesh). The filtrate was washed extensively by adding normal saline (0.85% NaCl), stirring vigorously and leaving it to stand for 30-60 minutes for sedimentation. The supernatant was then discarded and washing with normal saline. This washing process was repeated 8-9 times until the filtrate became clear. The sediment was centrifuged @ 3000 rpm for 5 min, then the supernatant was discarded and pellet was examined under stereomicroscope for metacercariae or metacestode.

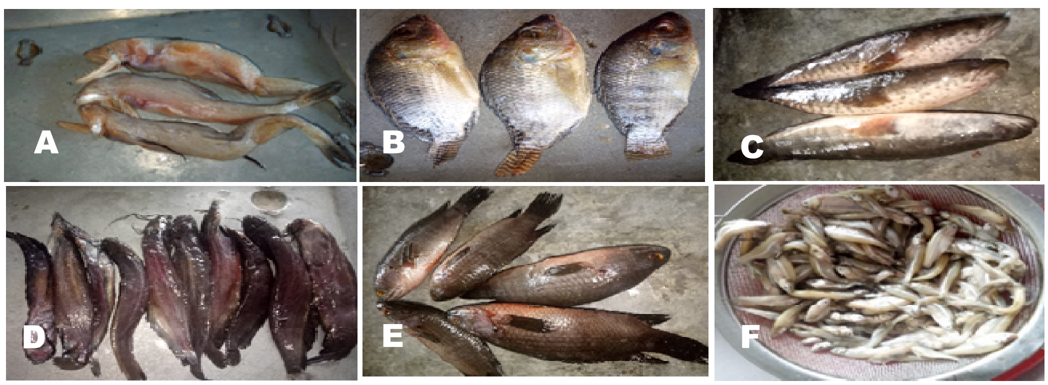

Fig. 2. Six type of freshwater fish samples were collected and examined in this study. (**A**) Loitta fish, (**B**) Tilapia, (**C**) Stinging cat fish, (**D**) Spotted snakehead fish, (**E**) Gangetic Koi and (**F**) Indian carplet fish.

***Identification of the metacercariae (MC)/metacestode*:** The pellet was re-suspended with fresh water and mixed thoroughly. A small drop of aliquot of the suspension was withdrawn with help of plastic dropper and put in a clean, grease free glass slide and examined under a microscope using 10X and 40X objectives. MC were identified to the genus level following the previously described keys and manuals (Sohn 2009, Kiyan *et al.* 2018, Knoff *et al.* 2011). Photographs of MC were taken using an inverted microscope to which a digital camera was fitted.

***DNA extraction and Polymerase Chain Reaction (PCR)*:** The genomic DNA was extracted directly from the metacercariae containing pellet using the QIAamp DNA Mini extraction kit (QIAGEN N.V Hilden, Germany) according to the manufacturer's instructions. The extracted DNA was then amplified using primers pair Anamika-F (5´-ATGACAATGTCGGTCGAAAGTA-3´) and Anamika-R (5´- CACATTAAGAAGGAGCCATTGC -3´) with a 360 bp (Rozas *et al.* 2012). PCR reactions were carried out in a 25 μl volume with 12.5 μl of Gotaq @ Green Master Mix (Promega Corporation, 2800 Woods Hollow Road, Madison, WI 53711-5399 USA), 1.5 μl of 10 pmol of each forward and reverse primer, 2.5 μl of genomic DNA template and 7 μl of deionized water, using a programmable automated thermocycler (Analytik Jena GmbH, Germany). The PCR program used involved an initial step 98°C for 10 min, followed by 35 cycles of 95°C for 1 min, 50°C for 1 min and 72°C for 1 min and a final extension step at 72°C 10 min. The amplified PCR products were analyzed by gel electrophoresis (2% agarose gel) stained with ethidium bromide and visualized the amplified PCR product under UV light using an image documentation system.

***Statistical analysis*:** Data were arranged in MS Excel Sheet and analyzed using the SPSS software. Prevalence and 95% confident intervals (CI) of fish-borne helminth parasite infections were calculated.

Results

***Morphological characteristics of different infective stages of fish borne zoonotic helminth parasites*:** The infective stages (metaceracariae and metacestodes) of processed samples were observed under microscope with 10X and 40X objectives after digestion with pepsin-HCl solution. Metacercariae and metacestode of *Opisthorchis* sp., *Clonorshis* sp., *Metagonimus* sp., *Metorchis* sp. and *Diphyllobothrium* spp. were identified based on the morphological characteristics described previously (Sohn 2009, Kiyan *et al.* 2018, Knoff *et al.* 2011). The morphological characteristics of the metacercariae of *Clonorchis* spp. were elliptical shape, there was presence of nearly equal sized oral and ventral suckers and brownish pigment granules that contains O-shaped excretory bladder. The morphology of *Opisthorchis* metacercariae were almost similar to those of *Clonorshis* sp. but the size was little bit bigger. In case of the plerocercoid of *Diphyllobothrium* sp., a shallow bothria was present at the anterior end and presence of introverted scolex. In case of *Metagonimus* sp., the metacercariae looks like a disc-shaped and presence of V-shaped excretory bladder. In case of *Metorchis* sp., it was globular in shape, there presence of double layered cyst wall and nearly equal sized oral and ventral suckers and O-shaped excretory bladder (Fig. 3).

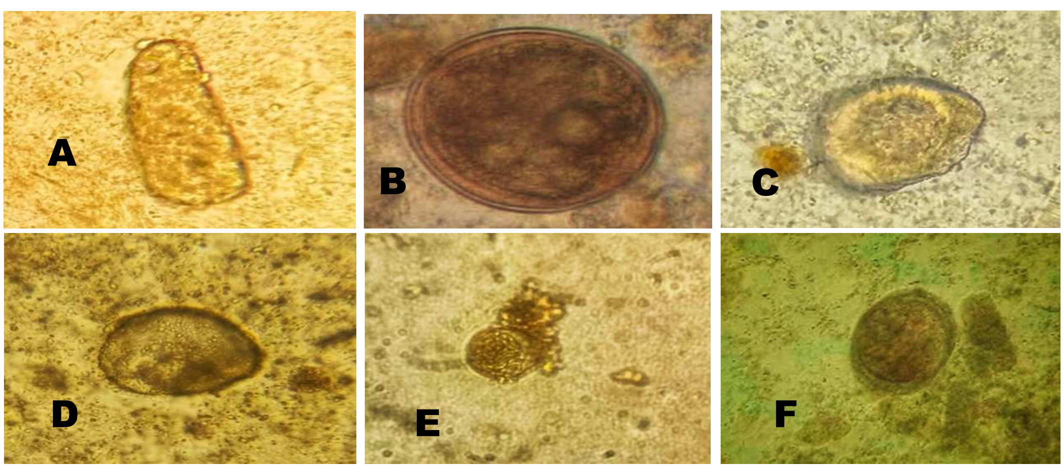

Fig. 3. Morphologically identified infective stages of fish-borne zoonotic parasites under microscope after pepsin HCL digestion. **(A)** Plerocercoid of *Diphyllobothrium* sp. (40X) from Loitta fish; **(B)** Metacercariae of *Clonorchis* sp. (40X) from spotted snakehead fish and stinging Cat fish **(C)** Metacercariae of *Opisthorchis* sp. (40X) from Loitta fish; **(D&E)** Metacercariae of *Metagonimus* sp. (40X,10X) from Loitta and Tilapia and **(F)** Metacercariae of *Metorchis orientalis* (40X) from Indian carplet fish.

***Prevalence of infective stages of FBZP in fishes*:** In microscopic examination, plerocercoid of *Diphyllobothrium* sp. from Loitta and Tilapia, metacercariae of *Clonorchis* sp. from Spotted snakehead and Stinging catfish, *Opisthorchis* sp. from Loitta fish, *Metagonimus* sp. from Loitta fish & Tilapia and *Metorchis orientalis* (Fig.3) from Indian carplet were identified based on their morphological characteristics described previously (Sohn 2009, Kiyan *et al.* 2018, Labony *et al.* 2020). Most of the fishes were found to be infected with multiple infective stages of helminth parasites such as *Opisthorchis* sp., *Clonorchis* sp., *Diphyllobothrium* sp. *Metagonimus* sp. and *Metorchis* sp. However, a single infection was found only in Indian carplet fish. About 58% overall prevalence of FBZPs was determined. Higher prevalence (80%) was found in Loitta fish followed by Spotted snakehead (65%), Tilapia (60%), Stinging catfish (45%) and Indian carplet (24.28%), respectively (Table I).

Table I. Prevalence of the infective stages (metacercariae/metacestode) of fish-borne zoonotic parasites in Bhola district of Bangladesh.

Fish Species	No. of fishes examined= n	No. of fishes infected= n	Prevalence of metacercaria /metacestode and 95% CI	Infective stages of helminth parasites
Bombay duck *(Harpadon nehereus)*	150	120	80% (72.89-85.62)	*Clonorchis / Opisthorchis/ Diphyllobothrium/ Metagonimus*
Tilapia *(Oreochromis nilolticus)*	25	15	60% (40.74-76.60)	*Metagonimus*
Spotted Snakehead *(Channa punctata)*	20	13	65% (43.29-81.88)	*Clonorchis / Opisthorchis*
Stinging catfish (*Heteropneustes fossilis*)	20	9	45% (25.82-65.79)	*Clonorchis / Opisthorchis*
Indian carplet (*Amblypharyngodon microlepis*)	70	17	24.28% (15.75-35.49)	*Metorchis*
Catla (*Catla catla*)	1	0	0	
Rohu *(Labeo rohita)*	2	0	0	
Silver carp (*Hypophthalmichthys molitrix*)	1	0	0	
Grass carp (*Ctenopharyngodon idella*)	1	0	0	
Climbing perch (*Anabas cobojius*)	10	0	0	
	Total=300	174		
	Overall Prevalence = 58% (174/300)			

***Molecular identification and confirmation of plerocercoid of Diphyllobothrium sp. from Loitta and Tilapia* fish:** The microscopic identification of plerocercoid, the developmental stage of *Diphyllobothrium* sp. were confirmed molecularly from the representative of highly positive samples of Loitta (n=2) and Tilapia (n=1) fishes by amplifying the partial fragment of 360 bp region of the 18S rRNA gene (Fig.4).

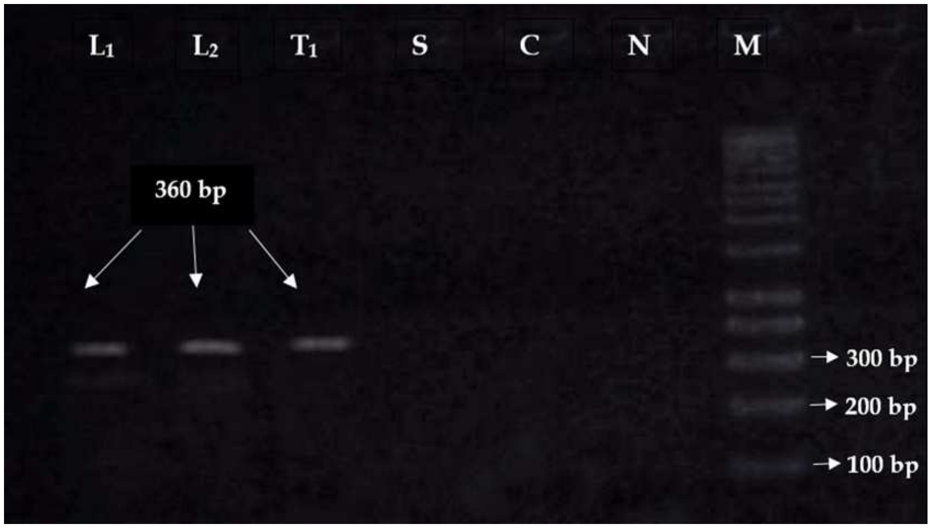

Fig. 4. Molecular identification and confirmation of *Diphyllobothrium* sp. by PCR. Amplified product of *Diphyllobothrium* sp. is 360 bp [M=Marker; N= Negative control; C= Cat fish; T_1= Tilapia; S= Snakehead fish; L_1+L_2= Loitta fish].

Discussions

Fish-borne zoonotic helminths parasites are one of the great important sources of diseases for human. It has a great public health impact worldwide. Due to scarcity of information on the geographical distribution and disease burden, their public health impact has been neglected for a long time. Various freshwater fishes, especially the cyprinid fishes act as second intermediate host of these zoonotic helminth parasites specially the cestode and trematode. In Bangladesh, there are a few scientific reports available regarding the fish-borne zoonotic helminth parasites in fresh water, wild and small fishes. This study focused on the Bhola district of Bangladesh that has a mixed water body of river and sea water. The harvested assorted fishes from this region are shifted to different markets throughout the country. Therefore, emphasis was given on the fishes from different local markets at Bhola district to explore the scenario of the important fish-borne zoonotic helminth parasites.

The present study revealed that five among ten types of wild fishes, namely, Loitta /Bombay duck (Family: Synodontidae), Tilapia (Family: Cichlidae), Spotted snakehead (Family: Channidae), Stinging cat fish (Family: Heteropneustidae) and Indian carplet (Family: Cyprinidae) were found to be positive with the infective stages of helminth parasites suggesting that these fishes play an important role as the second intermediate hosts to complete the life cycle of different fish-borne helminthes prevalent in the study area. Our results are similar with the results reported previously from different countries including Bangladesh showing that the cyprinid fishes are responsible for transmission of various fish-borne zoonotic trematodes (Petney *et al.* 2013, Conlan *et al.* 2011). In addition to cyprinid fishes, MC have also been identified from fresh-water garfish (Belonidae) in Thailand (Wongsawad *et al.* 2000) and Tilapia fish (family: Cichlidae) in Northern Vietnam (De, 2004) suggesting that fresh fishes can also act as the second intermediate hosts for FBZPs. It was reveals 58% overall prevalence of FBZP in fresh water fishes of Bhola district which are consistent with the results reported previously in the country where overall prevalence recorded 66% (Labony et al. 2020). Our findings are also in agreement with the earlier findings where 86.1% prevalence of metacercariae were detected in the wrestling halfbeak, *Dermogenys pusilla*, collected from Bangkok metropolitan region of Thailand (Patarwut *et al.* 2020). In our study, the MC of *Clonorchis/Opisthorchis* were detected from Loitta, spotted snakehead and stinging cat fish which suggests that these fishes act as the second intermediate hosts of opisthorchid flukes. On the other hand, our results are agreed with Labony *et al.* (2020), where the various MC of *Clonorchis/Opisthorchis* in ticto barb (*Puntius ticto*), banded gourami (*Colisa fasciata*), freshwater garfish (*Xenentodon cancila*), flying barb (*Esomus danricus*) and reba carp (*Cirrhinus reba*) have been detected from fresh water in Mymensingh and Kishoreganj district of Bangladesh. Although adult flukes were reported previously from the reservoir animals and birds indicating that these parasites were existed for a long time in the country (Shaikh and Huq 1984, Anisuzzaman *et al.* 2005). The people of the country seem to be at high risk with fish borne zoonotic helminth parasites since the fresh water fishes are in high demand both in rural and urban areas and are available throughout Bangladesh. It has been reported that *Opisthorchis viverrini* is considered the critical risk factor for the development of the bile duct cancer, cholangiocarcinoma (CCA), which is a major public health concern in Mekong region in Myanmar (Keiser and Utzinger 2009, Dorny *et al.* 2009, Sithithaworn *et al.* 2012, Pyo *et al.* 2013). The prevalence of *O. viverrini* was 10–29% in the crucian carp, *Carassius carassius*, in Phu Yen province (Chuong *et al.* 1997) and 1.9% in the snakehead fish, *Channa striata*, in An Giang province (Thu *et al.* 2007) in southern Vietnam is considered to be the '**hot spot**' of the parasite.

In the present study, the MC of *Metagonimus* spp. were identified from Loitta and Tilapia that is consistent with the previous results where MC of *Metagonimus* spp have been reported from several species of fresh water fishes such as ticto barb, banded gourami, freshwater garfish, and reba carp in Bangladesh (Labony *et al.* 2020) and *Abramis brama, A. ballerus, Aspius aspius, Blicca bjoerkna, C. carassius, Chondrostoma nasus, Hemibarbus labeo, Leuciscus idus, Pseudobagrus fulvidraco, Plecoglossus altivelis, Tribolodon taczanowskii (T. hakonensis),* and *Lateolabrax japonicus* from China, Taiwan, and Korea (Sohn, 2009; Yuan *et al.* 2018). Indian carplet was found to be positive for metacercariae of *Metorchis* spp. which is consistent with findings previously reported from ticto barb, banded gourami, freshwater garfish and reba carp in Bangladesh (Labony *et al.* 2020). However, MC of *Metorchis* spp. have been detected from humans in Eurasia, North America and East Asia (Lv *et al.* 2013). In contrast, *M. orientalis* has also been reported from ducks in Bangladesh (Anisuzzaman *et al.* 2005). The MC of *M. orientalis* were also detected in *Pseudorasbora parva, Hemiculter leucisculus, Saurogobio dabryi, Rhynchocypris lagowskii, C. auratus, Rhodeus ocellatus, Perccottus glehnii, P. herzi, Misgurnus anguillicaudatus, Microphysogobio koreensis* and *Gnathopogon strigatus* in China and Korea indicating they have a wide host preference (Qiu *et al.* 2017; Sohn *et al.* 2017).

The highest prevalence (80%) of FBZP was found in Loitta fish followed by Spotted snakehead (65%) and Tilapia (60%) and lowest prevalence was detected in Indian carplet fish (24.28%). The present findings are also in accordance with the findings reported in Bangladesh and Northern Vietnam respectively (Labony *et al.* 2020, De *et al.* 2012). We also detected the plerocercoid of *Dibothriocephalu* spp. from Loitta and Tilapia fishes and this finding is consistent with the findings reported from two catfishes Magur and Singhi in the marine and fresh water in Bangladesh (Chandra JK 2006). The variation of our present results from the previously reported results in and around Bangladesh may plausibly be due to different geographical regions, sample size and different types of fish species, geo-climatic condition, the environment, the water bodies and availability of the reservoir host.

In our study, the morphological identification of infective stages, plerocercoid of *Diphyllobothrium* sp. were detected from Loitta and Tilapia and confirmed by amplifying the fragment of 360 bp region of 18S rRNA which is the first time in Bangladesh. The present findings are in accordance with the findings of Rozas *et al.* (2012) in Chile where the authors confirmed the microscopic examination of plerocercoid of *D. dendriticum* by using PCR and sequencing. *Diphyllobothrium* spp. have worldwide distribution in various mammalian, piscine and avian hosts and it can be transmitted to the final host through ingestion of raw and undercooked freshwater fishes containing the infective stages, the plerocercoid. Here, we reported the first molecular identification of plerocercoid of *Diphyllobothrium* spp. in Loitta and Tilapia fishes from Bhola district, where it has not been previously detected. Advanced in molecular techniques are greatly improving the ability to detect this parasite in fish samples collected from various local market of Bhola district of Bangladesh. A detail studies are required with nationwide survey in order to get better understanding the epidemiology, life cycle, transmission and public health significance to formulate the control strategies against these fish-borne zoonotic helminth parasites including the Diphyllobrothiasis in Bangladesh. The results from the current study indicates that the riverine fishes are more likely to be infected with infective stages of various fish-borne zoonotic parasites due to the pollution and abundance of their second intermediate host. Since there are no control measures of snails thus, the fishes are continuously exposed to the cercariae released from snails (first intermediate host) and carry the infection as a second intermediate host to their final host. Therefore, awareness of local people should be increased about the danger of ingestion of undercooked fish dishes in order to prevent the transmission of fish-borne zoonotic helminth parasites.

Prevalence of fish-borne zoonotic helminth parasites are quite high in the study area indicating the endemicity of these parasites in the county. Hygienic measures and awareness of people should be increased for those who are fond of consuming the undercooked, smoked and raw fish and fish products. Herein, the morphological identification of plerocercoid of the zoonotic cestode (*Diphyllobothrium* sp.) is confirmed molecularly for the first time in Bangladesh. The present study will be a milestone for the future understanding of the genetic diversity and phylogeny of fish-borne zoonotic helminth parasites infections as well as be helpful to figure out the possible public health risk related to zoonoses in Bangladesh.

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