UNIVERSITI PUTRA MALAYSIA

MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF TREMATODE CERCARIA IN LYMNAEID SNAILS FROM SELECTED BUFFALO FARMS UNDER OIL PALM INTERGRATION IN PERAK, MALAYSIA

RHUBHITHRA A/P PUVANARAJAH

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2022/2023

CERTIFICATION

It is hereby certified that I have read this project paper entitled "Morphological and Molecular Identification of Trematode Cercaria in Lymnaeid Snails from selected buffalo farms under oil palm integration in Perak, Malaysia", by Rhubhithra A/P Puvanarajah and in your opinion, it is satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirement for the course VPD 4999 – Final Year Project.

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DEDICATIONS

To my parents,

Amma and Appa

for the unconditional love and care

To my brother

for always making me laugh

To my friends

for being the sunshine on my bad days

And to all the good souls who helped making this journey fun

ACKNOWLEDGMENTS

I would like to extend my deepest gratitude to my parents and my brother for believing in me and providing me support in many aspects.

I am highly indebted to my supervisor, Dr Nur Mahiza Md Isa and Dr Nazir Ahmad Tookhy for their guidance, supervision and patience throughout this project, despite of my shortcomings. It was an absolute honor to able to work with them. Thank you to the staff of Parasitology Laboratory for guiding me and providing me a comfortable learning space.

Lastly, thank you to my precious friends, Tanushah and Loh Jia Chi for constantly reminding me to never give up and providing me emotional support when I needed them.

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ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 – Projek Tahun Akhir

PENGENALAN MORFOLOGI DAN MOLEKUL CERCARIA TREMATOD DALAM SIPUT LYMNAEID DARI LADANG KERBAU TERPILIH DI BAWAH INTEGRASI KELAPA SAWIT DI PERAK, MALAYSIA

Oleh

Rhubhithra A/P Puvanarajah

2022

Penyelia: Dr Nur Mahiza Md Isa

Penyelia Bersama: Dr Mohd Mokhrish Md Ajat, Dr Reuben Sunil Kumar Sharma

Siput lymnaeid memainkan peranan penting dalam penularan cacing pipit parasit trematode yang menjangkiti haiwan dan manusia. Siput melepaskan cercaria trematod ke persekitaran, masuk ke dalam metacercaria yang merupakan tahap trematode yang infektif. Terdapat data terhad dan langka untuk cercaria trematod di Malaysia. Oleh itu, kajian ini adalah untuk menyelidiki ciri-ciri morfologi dan molekul cercaria trematod dalam siput Lymnaeid yang dikumpulkan dari beberapa ladang kerbau di bawah integrasi kelapa sawit di Perak. Sebanyak 876 sampel siput Lymnaeid dikumpulkan dan siput dihancurkan di antara dua slaid kaca untuk memerhatikan kemunculan cercaria trematod. Pengenalpastian trematod cercaria dilakukan oleh ciri-ciri morfologi cercaria. Selanjutnya, cercaria trematod yang diperoleh dikenal pasti secara molekul menggunakan teknik tindak balas berrantai polimerase (PCR). Kadar jangkitan oleh kemunculan cercaria trematod dari siput Lymnaeid adalah 8.73% (73/836). Berdasarkan ciri-ciri morfologi, empat jenis cercaria trematod dikenal pasti; *Echinostome, Xiphidiocercaria, Gymnocephalous dan Furcocercous cercaria. Echinostome cercaria* menunjukkan kadar jangkitan tertinggi dalam siput Lymnaeid 78.1% (57/73), diikuti oleh *Xiphidiocercaria* 26% (19/73). Kedua-dua *Gymnocephalous cercaria* dan *Furcocercous cercaria* mempunyai kadar jangkitan 4.1% (3/73). Analisis urutan yang menggunakan rantau spacer 2 transkrip dalaman (ITS2) menunjukkan tiga jenis morfologi cercaria tergolong dalam tiga spesies trematod; *Plagiorchis, Echinostome* dan *Fasciola*. Ketiga-tiga spesies trematod ini tergolong dalam trematoda digenean yang bersifat zoonotik. Ini adalah laporan pertama mengenai cercaria trematod zoonotik dalam siput Lymnaeid di Perak. Kesimpulannya, empat jenis cercaria trematod secara teknik morfologi dan tiga spesies cercaria trematod secara teknik molekul dikenal pasti dalam kajian ini. Oleh itu, adalah mustahak untuk mengawal populasi siput dalam sumber air untuk mencegah jangkitan cercaria dan seterusnya mencegah penyebaran trematod zoonotik di inang pasti.

Kata kunci: cercaria trematod zoonotik; ITS2; morfologi; Perak; Siput Lymnaeid

ABSTRACT

An abstract from the project paper presented to the Faculty of Veterinary Medicine in partial fulfillment of course VPD 4999 – Final Year Project

MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF TREMATODE CERCARIA IN LYMNAEID SNAILS FROM SELECTED BUFFALO FARMS UNDER OIL PALM INTEGRATION IN PERAK, MALAYSIA

By

Rhubhithra A/P Puvanarajah

2022

Supervisor: Dr Nur Mahiza Md Isa

Co-supervisors: Dr Mohd Mokhrish Md Ajat, Dr Reuben Sunil Kumar Sharma

Lymnaeid snails play an essential role in the transmission of trematode parasitic flatworms that can infect animals and humans. The snails release trematode cercaria into the environment, encysts into metacercaria which is the infective stage of trematode. There are limited and scarce data available on the trematode cercaria in Malaysia. Therefore, this study is to investigate the morphological and molecular characteristics of trematode cercaria in Lymnaeid snails collected from several buffalo farms under oil palm integration in Perak. A total of 876 Lymnaeid snail samples were collected, and the snails were crushed between two glass slides to observe the trematode cercarial emergence. The identification of trematode cercaria was done by the morphological characteristics of cercaria. Furthermore, the obtained cercaria were identified molecularly using polymerase chain reaction (PCR). The infection rate by trematode cercarial emergence in Lymnaeid snails was 8.73% (73/836). Based on morphological characteristics, four types of trematodes cercaria were identified: *Echinostome*, *Xiphidiocercaria*, *Gymnocephalous* and *Furcocercous cercaria*. *Echinostome cercaria* showed the highest infection rate in Lymnaeid snails 78.1% (57/73), followed by *Xiphidiocercaria* 26% (19/73). Both *Gymnocephalous cercaria* and *Furcocercous cercaria* had 4.1% (3/73) infection rate. The sequencing analysis of the cercarial internal transcribed spacer 2 (ITS2) showed three cercaria morphotypes belonged to three trematode species: *Plagiorchis*, *Echinostome* and *Fasciola*. These three trematode species belong to digenean trematode that are zoonotic. It is the first report on zoonotic trematode cercaria in Lymnaeid snails in Perak. In conclusion, four trematode cercaria types morphologically and three trematode cercaria species molecularly were identified in the present study. Hence, it is essential to control the snail population in water resources to prevent cercarial infection and further prevent the spread of zoonotic trematode in definitive host.

Keywords: ITS2; Lymnaeid snails; morphology; Perak; zoonotic trematode cercaria

1.0 INTRODUCTION

Trematodes, also known as flukes, are flatworm helminths that infect both animals and humans (Schell, 1970). Digenean trematodes belongs to one of the subclasses of Trematoda, are parasites that require two host organisms to complete their life cycle (Cribb et al., 2003). Freshwater snails act as an intermediate host, while a vertebrate act as a definitive host. Freshwater snails of *Lymnaeidae* family are of medical and veterinary importance since they are the vectors in transmission of digenean trematodes (Imani-Baran et al, 2013). According to Castro-Trejo (1990), there were five species of the genus Lymnaea found in ruminant farms, *L bullimoides, L collumella, L cubensis, L humilis* and *L palustris*. Inside of a lymnaeid snail, the larval development of a trematode takes place (sporocyst, redia and cercaria). Thus, a study on trematode larvae especially the cercaria in a lymnaeid snail could help prevent the transmission of trematode to the ruminants and humans, as some of them are zoonotic.

Trematode infections in ruminants such as Fasciolosis and Paramphistomosis, are transmitted by the trematode cercaria, causes the ruminants to lose weight, reduced milk production, infertility and eventually death (Hambal, 2020). As for humans, the most common disease is cercarial dermatitis, also known as the swimmer's itch, is a waterborne cutaneous allergic disease caused by a consequence of repeated infections of cercaria from Schistosoma trematode (Kolářová et al., 2013). Cercaria of a schistosome usually uses lymnaeid snail as the intermediate host. This implies that there is a correlation between trematode cercaria and the occurrence of trematode infections in the ruminant population, as well as the humans. Hence, it is important to correctly identify the species of trematode cercaria through morphological and molecular methods, as it provides a better understanding on the epidemiology of trematode infection and further develop preventive measures in the future.

There are several studies being done on the morphology and molecular characteristics of trematode cercaria from lymnaeid snails in Southeast Asian countries. Myanmar, one of the neighboring countries, reported to identify eight types of trematode cercaria in freshwater snails from a lake and a dam (Bawn et al., 2022). However, there is a lack of available studies done on the respective study in Malaysia. Therefore, the aim of this study was to characterize the morphological and molecular identification of trematode cercaria in Lymnaeid snail collected from buffalo farms under oil palm integration in Perak, Malaysia.

2.0 LITERATURE REVIEW

2.1 Lymnaeid Snail

Lymnaeid snails, commonly known as pond snails, are molluscs under *Lymnaeidae* family and *Lymnaeoidea* superfamily. Lymnaeids are distributed worldwide and serve as intermediate host for at least 71 species of trematodes from 13 different families, including *Paramphistomatidae*, *Echinostomatidae* and *Schistomatidae*, which is of veterinary importance (Bargues et al., 2001). A study by Correa (2010), there have been reported approximately 100 species of lymnaeids, some of which are *Lymnaea palustris*, *Lymnaea stagnalis* and *Lymnaea natalensis.* The most common trematode species transmitted by the lymnaeid snails is Fasciola hepatica. According to the same study, there are at least 20 species of lymnaeids acts as potential vectors for fasciolosis.

Lymnaeid snails are found globally inhabiting the rivers, lakes, swamps, streams, underground caves, springs, ditches, drainage as well as seasonal waters (Strong, 2007). The spatial distribution of lymnaeid snails have an impact on the prevalence of trematode infection. As the number of freshwater snails increases in the farm vicinity, the number of cercarial infection into the snails will increase (Bawn et al., 2022), thus a rise in trematode infection. Sokolow (2017) also supported that the increasingly population of the snails globally, particularly in areas where a dam or lake is located, snail-borne parasitic diseases could spread considerably rapid.

However, in Malaysia, one of the world's biodiversity hotspots, have studies emphasizing on the distribution of different snail species, but a very limited knowledge about snails and its contribution to trematode infection in veterinary aspect. In a recent study by Khadijah (2017), lymnaeid snails were found in waterlogged areas surrounding cattle farms situated in Terengganu, Malaysia.

2.2 Trematode Cercaria

Cercaria is a non-infective larval stage of Trematoda class. In simple, cercaria morphology compromised of a body, with one or two suckers, and a tail used to propel through water (Morley., 2012). The distribution of internal organs in all cercariae, regardless of species, usually resembles that of the adult worm. The oral suckers are usually located at the anterior part, while the ventral suckers are located at posterior or mid-ventral part of the body (Frandsen and Christensen, 1984). Cercaria has an alimentary canal which consists of mouth, pharynx, esophagus, esophageal glands, and a pair of intestinal caeca. The contents of these glands are secreted during the cercarial emergence from a snail intermediate host. Apart from that, cercaria also has its own excretory system which consists of an excretory bladder and collecting tubes. The arrangement of excretory cells of a cercaria are similar to its adult form. Thus, if the excretory cell pattern is identified, then it can be used to correlate the larval form with its adult stage (Frandsen and Christensen, 1984).

2.3 Role of Cercaria in Life Cycle of Trematodes

Trematodes require an intermediate host and a definitive host to complete the life cycle. Molluscs like Lymnaeid snails are the intermediate host, while mammals including humans are the definitive host. **Figure1** shows the life cycle of a digenean trematode and the role of a cercaria in developing the larval stage of trematode. The life cycle of trematode begins when the adult fluke shed trematode eggs in the stomach of an infected definitive host and the eggs are passed through faeces into the environment. The eggs in the pastures hatched under adequate moisture and temperature, to release miracidia within 24 hours (Waal et al, 2010). Motile miracidia infects a mollusc to develop into sporocyst, followed by redia, then into cercaria. The role of a cercaria comes to play when they have a high specificity in host-finding and host-recognition, which makes a cercaria to invade actively a spectrum of specific host (Haas et al, 1994). This unique behavioral pattern maximizes the success rate of transmission of trematode. Cercaria emerges out of the mollusc by the stimulation of sunlight, particularly at 10°C to 26°C (Kendall & McCullough, 1951) and encysts into metacercaria on herbages. Metacercariae are ingested from pasture by grazing livestock and undergo excystment within the small intestine (John et al., 2019). Metacercaria excysts when a grazing livestock ingests contaminated herbages to hatch juvenile fluke. The juvenile fluke then migrates, attaches to small intestine, where maturation, reproduction and egg laying occur, re-initiating the parasitic life cycle (John et al., 2019).

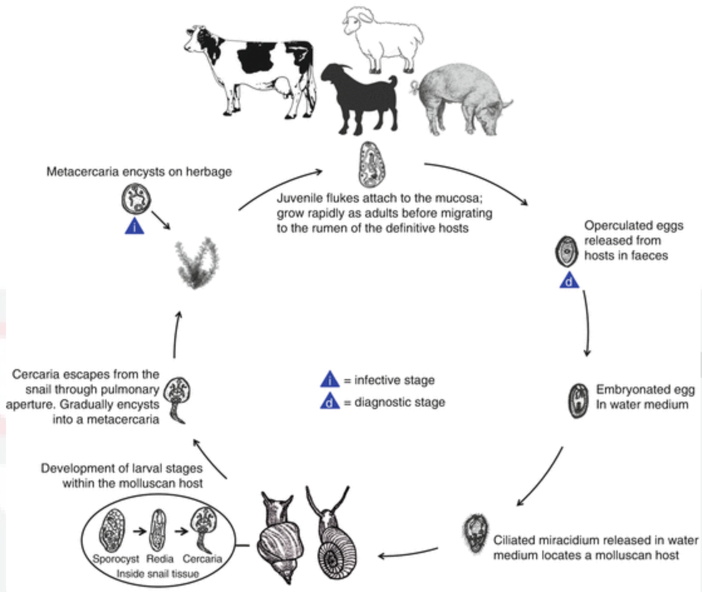

Figure 1: Life Cycle of Trematode

2.4 Identification of Trematode Cercaria

2.4.1 Morphological Identification

Morphology of a trematode cercaria is important to identify accurately, as there are species of cercaria carries zoonotic trematodes which could poses a risk to public health. A standardized taxonomic key described by Frandsen and Christensen (1984) is being widely used to identify cercaria morphologically. There are two classifications of cercaria; major types and minor sub-types. To classify the cercaria to its major types, the morphological characteristics that needed to determine are position of body suckers, shape of cercarial tails and the presence or absence of specialized structures such as stylet. As for the sub-types of cercaria, minor differences in structures such as presence of virgula organs and spiny collar, provides a detailed identity.

Issues tend to arise regarding identification of cercaria at the species level as the observation of gross morphology only provides identification at family level and occasionally to the genus level. Hence, biological characteristics of cercaria is considered to be effective method to identify cercaria at the species level, along with gross morphology identification. Biological characteristics of cercaria are such as cercarial developmental stages, cercarial swimming behavior and cercarial resting position. However, an accurate identification of cercaria at the species level may not achieved with gross morphology and biological characteristics, as it is subjective, and it requires expertise.

2.4.2 Molecular Identification

Molecular identification of trematode cercaria is an efficient and accurate method compared to morphological identification of cercaria at a higher resolution, which is at the species level. Internal transcribed spacer 2 (ITS2) region of the 18S rDNA gene is an established molecular marker that have been used widely for identification of various stages in the life cycle of trematode, including cercaria (Esteban et al. 2014, Blasco-Costa et al. 2016, Chai 2019). Conventional PCR method is utilized in identifying cercaria as the nuclear DNA method is highly accurate, sensitive and rapid. However, conventional PCR method was found to be ineffective in identifying *Furcocercous cercaria* due to poor PCR amplification of targeted genes. Therefore, semi-nest PCR method targeting 28S rRNA gene was chosen to identify *Furcocercous cercaria.* In general, ITS2 of 18S rDNA gene is a reliable molecular marker in distinguishing a wide variety of cercaria species.

3.0 MATERIALS AND METHODS

3.1 Sampling Location

The sampling location was situated at two buffalo farms and three surrounding wetlands in Temoh, Perak, Malaysia (**Figure 2**).

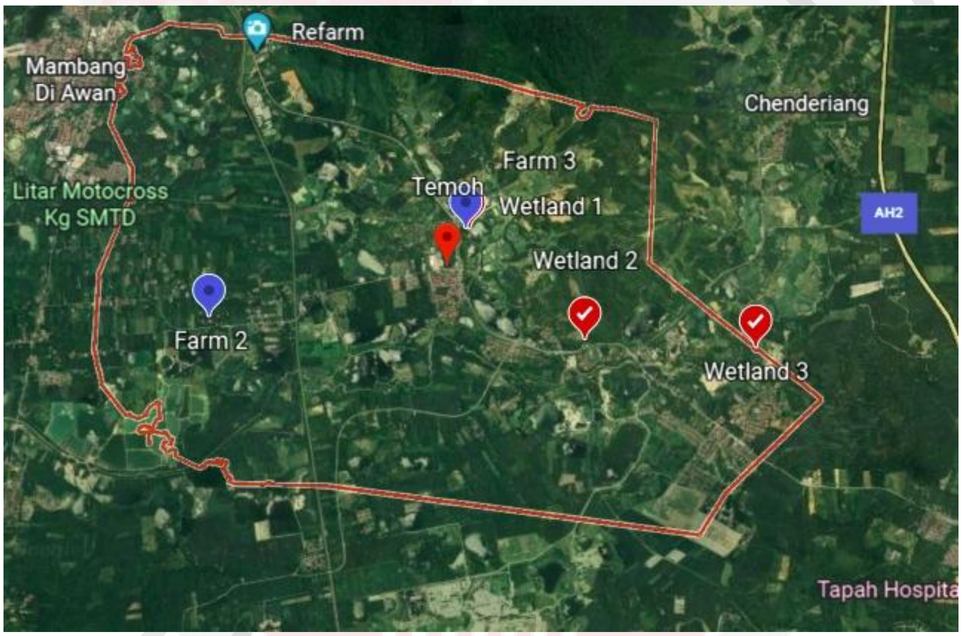

Figure 2: Sampling location at buffalo farms and surrounding wetlands in Temoh, Perak, Malaysia.

GPS Coordinates:

Farm 2: 4°14’53°N 101°11’20 °E

Farm 3: 4°14’53°N 101°11’48°E

Wetland 1 (adjacent to Farm 3): 4°14’53°N 101°11’49°E

Wetland 2: 4°14’05°N 101°12’41°E

Wetland 3: 4°14’02°N 101°13’54°E

The sampling locations are shallow waterlogged areas with presence of Lymnaeid snials (**Figure 3**).

Figure 3: Shallow waterlogged areas with Lymnaeid snails present.

3.2 Sampling Size and Method

This study was able to conduct with the approval of Institutional Animal Care and Use Committee (IACUC) with the reference number: UPM/IACUC/AUP-U046/2022. The sampling method used to collect Lymnaeid snails was random sampling. Sample collection was done using a scoop net (**Figure 4**) for 20 minutes at each site between 8:00 am to 11:00 am, performed by the same person. The collected samples were placed inside a 45ml capacity container and closed it with perforated cover (**Figure 5**), to provide aeration for the snails. Each container was filled with approximately 10ml of water from the habitats. The samples were then transported to Parasitology Laboratory, Faculty of Veterinary Medicine for further process.

Figure 4: Scoop net containing with snail samples

Figure 5: Storage of snail samples in 45ml capacity container

3.3 Morphological Identification of Trematode Cercaria

Snails were crushed using two glass slides to allow the emergence of cercaria. The live cercaria was examined under both stereomicroscope and light microscope using 10x magnification. The live cercaria was then stained using Ehrlich's hematoxylin stain to obtained stained preserved cercaria. Each stained cercaria was examined under light microscope using 10x magnification. Morphological identification of the cercaria was performed using a standardized taxonomic key described by Frandsen and Christensen (1984) was referred. Pictures of cercaria viewed under light microscope were taken to identify the morphology of cercaria.

3.4 Molecular Identification of Trematode Cercaria

3.4.1 DNA Extraction

Using a DNeasy Blood and Tissue kit (QIAGEN, Hilden, Germany), a total of twelve genomic DNA were extracted from four morphologically similar cercaria groups from Lymnaeid snails. 5 µl of each cercaria samples were drop into a 1.5 m microcentrifuge tube. 180 µl of Buffer ATL was added into each tube, followed by 20 µl of proteinase K. The solutions were mixed by vortexing and incubated at 56℃ water baths until completely lysed. The microcentrifuge tubes were vortexed occasionally during the incubation process. Once completely lysed, the samples were vortexed again directly for 15 s. Then, 200 µl of Buffer AL was added and mixed thoroughly by vortexing followed by incubation at 56°C water baths for 10 min. Subsequently, 200 µl of ethanol (96-100%) was added and mixed thorougly by vortexing. The mixture was then pipetted into a DNeasy Mini spin column placed in 2 ml collection tubes. The collection tubes were centrifuged at 6000 x g (8000 rpm) for 1 min. The flow through and collection tubes were discarded, and the spin column was placed in new 2 ml collection tubes. 500 µl of Buffer AW1 was added into the 2 ml collection tubes and centrifuged at 6000 x g (8000 rpm) for 1 min. The previous step was repeated by discarding the flow through and collection tubes, followed by placing the spin column in new 2 ml collection tubes. Next, 500 µl of Buffer AW2 was added into the 2 ml collection tubes and centrifuged at 20,000 x g (14,000 rpm) for 3 min. The flow through and the collection tubes were discarded. The spin column is transferred to new 2 ml microcentrifuge tubes. The DNA was eluted by adding 200 µl Buffer AE to the center of the spin column membrane followed by incubation at room temperature (15-25°C) for 1 min. The microcentrifuge tubes were centrifuged for 1 min at 6000 x g (8000 rpm). The extracted DNA samples were stored at -80°C until used for PCR technique.

3.4.2 Polymerase Chain Reaction

The internal transcribed spacer 2 (ITS2) region was chosen for molecular identification of digenean trematode species because this region has been proven to be a reliable marker (Esteban et al. 2014, Blasco-Costa et al. 2016, Chai 2019). The ITS2 region of trematodes was amplified using a pair of primers described by Barber et al. (2000), consisting of a forward primer, ITS3 (5′-GCA TCG ATG AAG AAC GCA GC-3′), and a reverse primer, ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′). Total volume of 25 µl per each 1.5 ml microcentrifuge tube was used for amplification consisting of 10.6 µl master mix, 8.4 µl deionized distilled water, 4 µl DNA template, 1 µl forward primer (5′-GCA TCG ATG AAG AAC GCA GC-3′), and 1 µl reverse primer (5′-TCC TCC GCT TAT TGA TAT GC-3′). The master mix contains Taq DNA Polymerase, PCR buffer and dNTPs. A negative control was included containing deionized distilled water as a substitute for the master mix while no positive control was included in the amplification process. Thermal cycling was performed with an initial denaturation at 94℃ for 4 mins, followed by denaturation at 98℃ for 10 s, annealing at 50℃ for 15 s, 30 cycles of extension at 68℃ for 1 min and a final extension at 68℃ for 5 mins.

3.4.3 Gel Electrophoresis and UV Illumination

The protocol of a big sample was used for 0.75% agarose gel preparation. 1.5 g of Hydrogel powder was added into 100 ml of Tris-acetate-EDTA (TAE) buffer solution and 5 µl of RedSafe™ Nucleic Acid

Staining Solution was used for staining purpose. The gel was poured inside the castel and left it harden for 30 mins. 8 µl of each PCR products were placed in the wells starting from third well to tenth well. The second well was for negative control where 8 µl of deionized distilled water was added. A 5 µl of DNA ladder and 1 µl of loading dye were filled into the first well. The agarose gel was then run in the electrophoresis machine at 80 V and 400 Ma for 50 mins. Once the electrophoresis was completed, the agarose gel was read using short UV wave illumination in an Ultraviolet (UV) transilluminator to view the DNA bands.

3.4.4 DNA Sequencing and Bioinformatics Analysis of Gene Sequence

The obtained samples and primers were sent to First Base Laboratory Sdn Bhd for sequencing using the Sanger sequencing method. The sequences received were analyzed using BLAST® Standard Nucleotide (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine the percentage of homologous of the gene. The sequences were further referred to GenBank® sequence database to determine the similarity of the samples to the available sequences.

RESULTS

4.1 Morphological Analysis of Trematode Cercaria

In this study, a total of 876 Lymnaeid snails that were collected from the sampling sites, while 836 Lymnaeid snails were examined for the morphological identification of trematode cercaria (**Table 1**). There were 73 Lymnaeid snails showed positive cercarial emergence upon crushing method. The overall prevalence of cercarial infection was found to be 8.73% (73/836). According to the morphological examination, four distinct cercaria groups were identified, namely *Echinostome cercaria, Xiphidiocercaria cercaria*, *Gymnocephalous cercaria* and *Furcocercous cercaria*. The criterion for each type of cercaria groups were referred to the standardized taxonomic key described by Frandsen and Christensen (1984).

Table 1: Prevalence of cercarial infection in each snail collected from wetlands located at Temoh, Perak

No	Study area	Snail family	No. of collected snail	No. of died snail	No. of examined snail	No. of infected snail	No of infected snail samples with cercaria species				Prevalence % (No. of infected snail/ No. of examined snail)
							Ech	Xip	Gym	Fur	
1	Wetland 1	Lymnaeidae	262	11	251	51	47	8	0	1	20.32 (51/251)
2	Wetland 2	Lymnaeidae	325	15	310	13	5	7	2	0	4.19 (13/310)
3	Wetland 3	Lymnaeidae	289	14	275	9	5	4	1	2	3.27 (9/275)
	Total		876	40	836	73	57	19	3	3	8.73 (73/836)

Ech: *Echinostome cercaria*, Xip: *Xiphidiocercaria*, Gym: *Gymnocephalous cercaria*, Fur: *Furcocercous cercaria*

4.1.1 *Echinostome cercaria*

Echinostome cercaria was found in 57 infected Lymnaeid snails and had the highest prevalence rate by cercarial emergence among the four types of cercaria groups at 78.1%. The body of cercaria is elongated in shape. Oral sucker is located at anterior surface of the body, circular in shape and surrounded by spiny collar. Ventral sucker is located at mid-ventral surface of the body. The tail is slender, unforked, and as long or longer than the body (**Figure 6**).

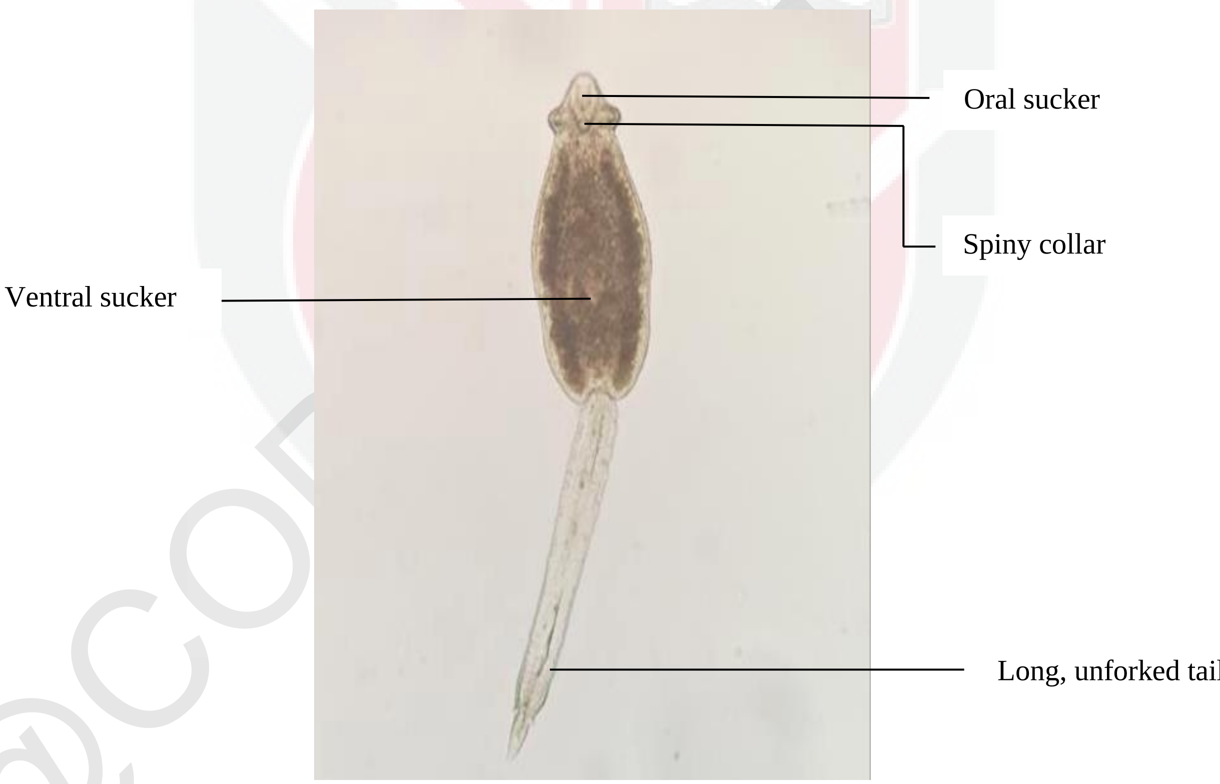

Figure 6: *Echinostome cercaria*, (10x light microscope)

4.1.2 *Xiphidiocercaria cercaria*

The body of cercaria is oval. Oral sucker is located at anterior surface of the body, circular in shape and consists of stylet. Ventral sucker is located at mid-ventral surface of body. Tail is unforked. There are three different subtypes under *Xiphidiocercaria cercaria* group. The *Xiphidiocercaria cercaria* that were identified in this study is *Virgulate xiphidiocercaria* (**Figure 7**). The characteristics of this cercaria including absence of dorsoventral finfold tail, virgula organ in the oral sucker and the size of ventral sucker is smaller than oral sucker. Out of 73 Lymnaeid snails, 26% were infected with this subtype of cercaria.

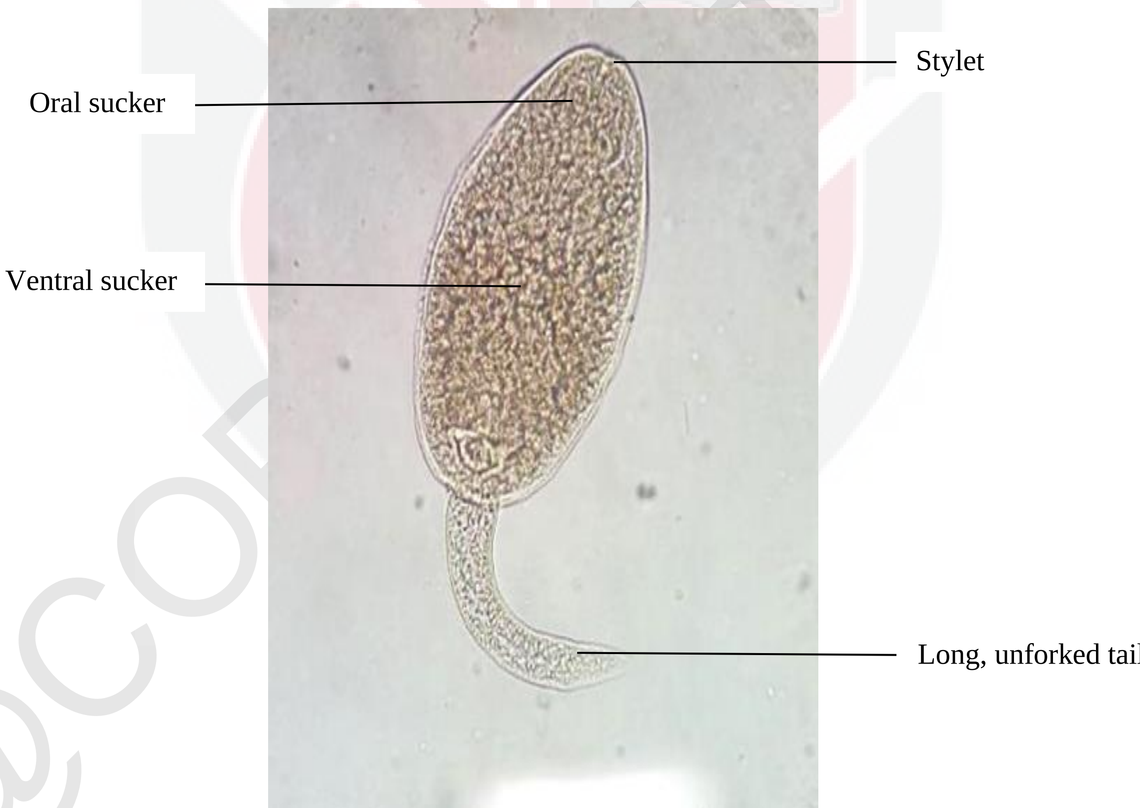

Figure 7: *Virgulate xiphidiocercaria*, (10x light microscope)

4.1.3 *Gymnocephalous cercaria*

The body of cercaria is circular in shape. Oral sucker is located at anterior surface of the body, and neither stylet nor spiny collar present. Ventral sucker is located at mid-ventral surface of the body. Tail is unforked and it is longer than the body (**Figure 8**). 4.1% of this cercaria type was found in this study.

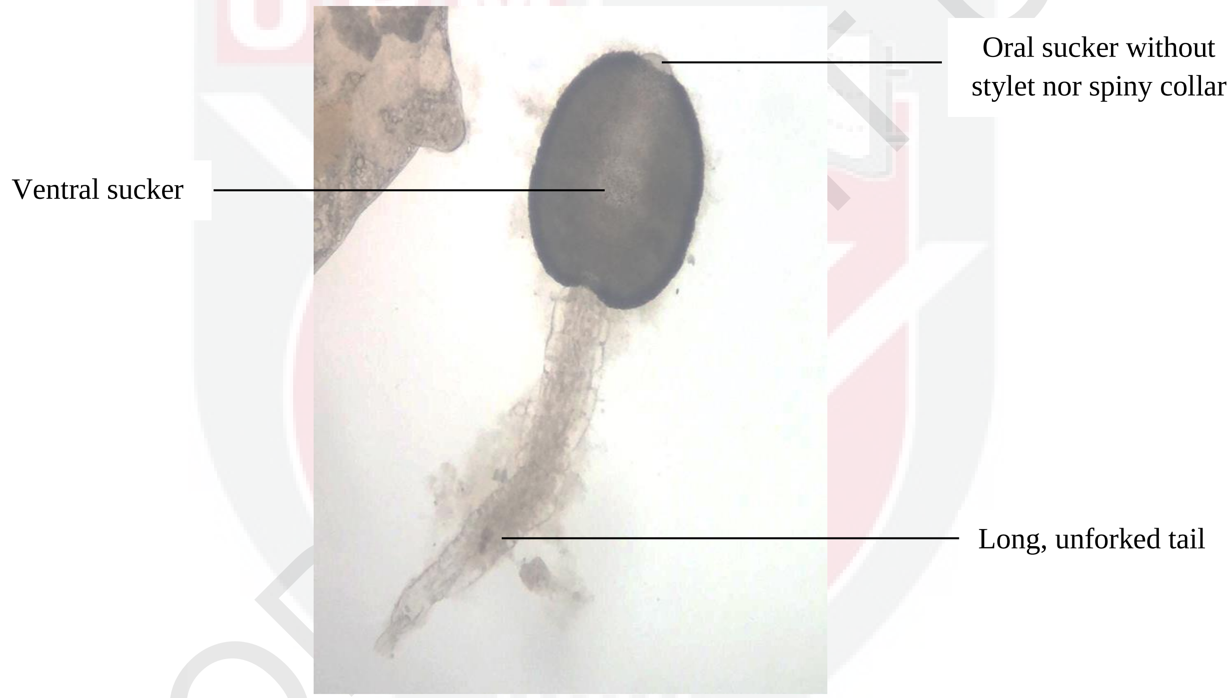

Figure 8: *Gymnocephalous cercaria,* (10x light microscope)

4.1.4 *Furcocercous cercaria*

Similar to *Gymnocephalous cercaria*, this cercaria type had 4.1% of prevalence rate in the Lymnaeid snails. One of the common characteristics of this cercaria type is the forked tail. There are four subtypes under this cercaria type which can be determined by two criterias; the type of furcation of the tail, and the presence or absence of suckers. For this study, the cercaria had long, forked with brevifurcate tail, and present of ventral sucker. Based on these morphological criteria, this is a *Brevifurcate-apharyngeate distome cercaria* (**Figure 9**).

Figure 9: *Brevifurcate-apharyngeate distome cercaria*, (10x light microscope)

4.2 Molecular Analysis

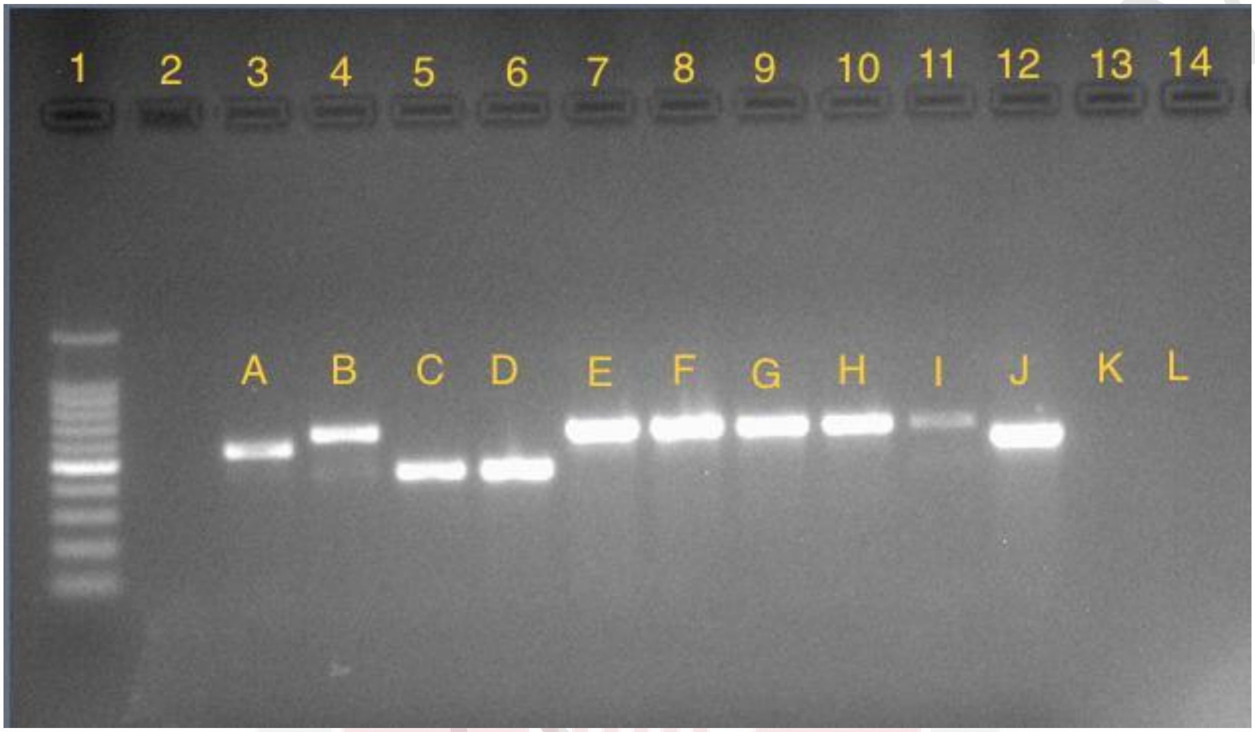

Figure 10: DNA bands of cercaria morphotypes samples from Lymnaeid snail

Lane 1: 500 bp DNA marker

Lane 2: negative control

Lane 3-14: samples from four different group of cercaria morphotypes

A-D: samples from *Virgulate xiphidiocercaria* morphotype

E-H: samples from *Echinostome cercaria* morphotype

I&J: samples from *Gymnocephalous cercaria* morphotype

K&L: samples from *Brevifurcate-apharyngeate distome cercaria* morphotype

4.2.1 Bioinformatics Analysis of Gene Sequence

The ten samples with bands formed were sent to First Base Laboratory for sequencing purposes. No bands were formed for sample K and L from *Brevifurcate-apharyngeate distome cercaria* morphotype, hence no sequence was received. The reference isolates from GenBank® database using the BLAST® Standard Nucleotide application showed 90 to 100% homologous with all ten sequences that were obtained. For sequences obtained from all four *Virgulate Xiphidiocercaria* morphotypes, the DNA gene showed that they belong to *Plagiorchis sp.* which are identical with isolates from Germany (KX160475.1, KX160474.1) and Czech Republic (KJ533391.1) with different percentage of homologous as seen in **Table 2.**

Table 2: Reference isolates from GenBank database that are identical with samples from *Virgulate Xiphidiocercaria* morphotype (A, B, C and D)

Description of Isolates	Percentage of homologous (%)				Accession Number
	A	B	C	D	
Plagiorchis sp. 4 DSG-2016 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence (Germany)	96.79	97.01	97.02	97.02	KX160475.1
Plagiorchis sp. 3 DSG-2016 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence (Germany)	96.79	97.01	97.02	97.02	KX160474.1
Plagiorchis maculosus isolate LSB12 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence (Czech Republic)	97.19	97.43	97.44	97.44	KJ533391.1

For sequences obtained from all four *Echinostome cercaria* morphotypes, the DNA gene showed that they belong to *Echinoparyphium sp.* which are identical with isolates from Lithuania (KJ542640.1) and *Echinostoma sp.* from Russia and Thailand, with accession number MZ517175.1 and MW199188.1 respectively as seen in **Table 3** with 91 to 92% of homologous.

Table 3: Reference isolates from GenBank database that are identical with samples from *Echinostome cercaria* morphotype (E, F, G and H)

Description of Isolates	Percentage of homologous (%)				Accession Number
	E	F	G	H	
Echinoparyphium mordvilkowi 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence (Lithuania)	92.45	92.47	92.64	92.45	KJ542640.1
Echinostoma bolschewense voucher TDre28/1 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence (Russia)	92.71	92.72	92.73	92.55	MZ517175.1
Echinostoma miyagawai isolate EMI2 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence (Thailand)	92.14	92.15	92.15	91.97	MW199188.1

For sequences obtained from two *Gymnocephalous cercaria* morphotypes, the DNA gene showed that they belong to *Fasciola gigantica* which are identical with isolates from Vietnam (MT429177.1, MT429174.1 and MN970009.1) with 99.61 % and 99.42% of homologous for sample I and J as seen in **Table 4**.

Table 4: Reference isolates from GenBank database that are identical with samples from *Gymnocephalous cercaria* morphotype (I and J)

Description of Isolates	Percentage of homologous (%)		Accession Number
	I	J	
Fasciola gigantica isolate Fas8 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence (Vietnam)	99.61	99.42	MT429177.1
Fasciola gigantica isolate Fas5 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence (Vietnam)	99.61	99.42	MT429174.1
Fasciola gigantica isolate NB small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence (Vietnam)	99.61	99.42	MN970009.1

5.0 DISCUSSION

The current study is the first report on the infection rate by cercarial emergence in Lymnaeid snails collected from buffalo farms under oil palm integration located in Perak. The overall prevalence of cercarial infection in Lymnaeid snails in this study was 8.73%. In contrast, reports from Southeast Asia countries showed different prevalence rate of cercarial infection in snails compared to the current study. In Indonesia, there was an overall infection rate of 3.75% (Prastowo et al. 2022), while in Thailand, the overall infection rate was 5.9% (Anucherngchai et al. 2016). In Vietnam, the overall prevalence of cercarial infection was 3.7 to 5.5%. In Myanmar, 5.8% of prevalence rate of cercarial infection in freshwater snails were reported (Bawm et al. 2022). The current study showed slightly higher prevalence rate of cercarial infection compared to the neighboring countries. The reason for this could be that the study sites were home to a variety of water resources and buffalo farms that are integrated with oil palm plantation, making this an ideal environment for cercaria to be effectively transmitted from snails to buffalo, allowing their life cycles to be perpetuated.

Based on morphological and molecular examination, three out of four cercaria morphotypes were molecularly classified into three digenean trematode species that are also zoonotic. *Plagiorchis sp.* which were extracted from DNA of *Virgulate Xiphidiocercaria* is a gastrointestinal fluke that is zoonotic when humans ingest the second intermediate host, freshwater fish. The *Echinostome cercaria* group carried *Echinostoma sp.* which is a gastrointestinal fluke that infects reptiles, birds and mammals. It is a zoonotic trematode when human ingest freshwater snails that were infected with metacercaria. *Gymnocephalous cercaria* that carried *Fasciola gigantica* in the Lymnaeid snails is a liver fluke that infects ruminants. Humans are susceptible to Fasciolosis when contaminated watercress with metacercaria being ingested. These findings suggest that the Lymnaeid snails in Temoh, Perak are exposed to trematode infection, which poses a health risk to the locals.

6.0 CONCLUSION

As a conclusion, cercaria that infected Lymnaeid snails collected from buffalo farms under oil palm intergration in Perak, Malaysia had four morphologically similar cercaria groups; *Echinostome cercaria*, *Virgulate Xiphidiocercaria*, *Gymnocephalous cercaria* and *Brevifurcate-apharyngeate distome cercaria* respectively. Sequencing analysis of cercarial ITS2 sequences revealed that four cercaria morphotypes were belonged to three digenean trematode species; *Plagiorchis sp*, *Echinostoma sp.* and *Fasciola gigantica*, which all of them are zoonotic trematodes.

7.0 RECOMMENDATIONS

Future research on the morphological and molecular identification of trematode cercaria in Lymnaeid snails at other locations in Malaysia with larger sample size should be done. Number of cercarial dermatitis cases among the locals should be recorded, as these cercariae are zoonotic and the disease might have an impact on the livelihood of the locals.

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APPENDIX A

List of materials and equipment for sampling and sample storage

List	**Amount**
Materials	
Gloves	A few pairs
45 ml capacity container	1 plastic
Test tubes rack	10
Scoop net	1
Perforated plastic	5 plastics
Rubber bands	1 plastic
Equipment	
Storage unit	-80℃

APPENDIX B

List of materials and equipment for morphological identification

List	Amount
Materials	
1.5 ml microcentrifuge tubes	1 jar
Microcentrifuge tube rack	5
Glass slides	20
Cover slips	20
Disposable plastic pipettes	2
Ehrlich's hematoxylin stain	5 ml
Equipment	
Light microscope Kern OBT-1	1
Stereomicroscope Nikon ECLIPSE E200	1

APPENDIX C

List of materials and equipment for DNA extraction

List	**Amount**
Materials	
Water bath	56℃
Proteinase K	20 µl
Buffer ATL	180 µl
Buffer AL	200 µl
Cercaria sample	5 µl
Ethanol	96-100%
Buffer AW1	500 µl
Buffer AW2	500 µl
Buffer AE	200 µl
Equipment	
1.5 ml of microcentrifuge tubes	1 jar
Microcentrifuge tube rack	1
Pipette (10, 100, 1000 µl)	1
Pipet tips (10, 100, 1000 µl)	3 boxes
2 ml collection tubes	1 plastic
DNeasy mini spin column	1 plastic
Centrifuge machine	1
Incubator	1
Vortexer	1
Storage unit	-80℃

APPENDIX D

Protocol for DNA Extraction using DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany)

1. Place 5 µl of each cercaria samples into a 1.5 ml microcentrifuge tube.
2. Add 180 µl of Buffer ATL into each tube.
3. Add 20 µl of proteinase K, mix by vortexing and incubate at 56℃ water baths until completely lysed.
4. Vortex the microcentrifuge tubes occasionally during the incubation process.
5. Once completely lysed, vortex the samples again directly for 15 s. before proceeding to next step.
6. Add 200 µl of Buffer AL. Mix thoroughly by vortexing. Incubate the samples at 56°C water baths for 10 min.
7. Add 200 µl of ethanol (96-100%). Mix thoroughly by vortexing.
8. Pipet the mixture into a DNeasy Mini spin column placed in 2 ml collection tubes. Centrifuge at ≥6000 x g (8000 rpm) for 1 min. Discard the flow-through and collection tubes.
9. Place the spin column in new 2 ml collection tubes. Add 500 µl of Buffer AW1. Centrifuge at ≥6000 x g (8000 rpm) for 1 min. Discard the flow-through and collection tubes.
10. Place the spin column in new 2 ml collection tubes. Add 500 µl of Buffer AW2. Centrifuge at 20,000 x g (14,000 rpm) for 3 min. Discard the flow-through and collection tubes.
11. Transfer the spin column to new 2 ml microcentrifuge tubes.
12. Elute the DNA by adding 200 µl Buffer AE to the center of the spin column membrane. Incubate at room temperature (15-25°C) for 1 min. Centrifuge the microcentrifuge tubes for 1 min at ≥6000 x g (8000 rpm).
13. Store the extracted DNA samples at -80°C until used for PCR technique.

APPENDIX E

List of materials and equipments for Polymerase Chain Reaction (PCR) technique

Lists	Amount
Materials	
DNA templates	4 µl
Deionized distilled water (Negative control)	8.4 µl
Master Mix	10.6 µl
Forward primer (5′-GCA TCG ATG AAG AAC GCA GC-3′)	12 µl
Reverse primer (5′-TCC TCC GCT TAT TGA TAT GC-3′)	12 µl
Equipments	
1.5 ml of microcentrifuge tubes	1 jar
Microcentrifuge tube rack	1
Pipette (10, 100, 1000 µl)	1
Pipet tips (10, 100, 1000 µl)	3 boxes
PCR Thermal cycler	1

APPENDIX F

Protocol for Polymerase Chain Reaction (PCR) technique using ITS2 region

1. Place 12 of 1.5 ml microcentrifuge tubes in a microcentrifuge tube rack.
2. Add 4 µl of DNA template into each 1.5 ml microcentrifuge tubes using a pipette.
3. Add 8.4 µl deionized distilled water into each 1.5 ml microcentrifuge tubes using a pipette.
4. Add 10.6 µl master mix into each 1.5 ml microcentrifuge tubes using a pipette.
5. Add 12 µl forward primer (5′-GCA TCG ATG AAG AAC GCA GC-3′), and 12 µl reverse primer (5′-TCC TCC GCT TAT TGA TAT GC-3′) into each 1.5 ml microcentrifuge tubes using a pipette.
6. Using a PCR thermal cycler, set up an initial denaturation at 94℃ for 4 min, denaturation at 98℃ for 10 s, annealing at 50℃ for 15 s, 30 cycles of extension at 68℃ for 1 min and a final extension at 68℃ for 5 min.

APPENDIX G

List of materials and equipments for gel electrophoresis and UV illumination

Lists	Amount
Materials	
Hydrogel powder	1.5 g
Tris-acetate-EDTA (TAE) Buffer	100 ml
RedSafe™ Nucleic Acid Staining Solution	5 µl
DNA ladder	5 µl
Dye	1 µl
Deionized distilled water (Negative control)	8 µl
Amplified PCR products (Samples)	8 µl
Equipments	
Flask	1
Microwave	1
Well comb	1
Casting tray	1
Electrophoresis unit	1
UV Transilluminator	1
Parafilm	1

APPENDIX H

Protocol for Gel Electrophoresis and UV Illumination

1. Measure 1.5 g of Hydrogel powder using a weighing scale and add into flask.
2. Add 100 ml of Tris-acetate-EDTA (TAE) buffer solution into a flask and mix.
3. Heat the gel in a microwave for 1 min to mix thoroughly.
4. Cool down the gel in a room temperature for 3 min.
5. Add 5 µl of RedSafe™ Nucleic Acid Staining Solution into the bottle and mix.
6. Pour the gel into the casting tray with well comb and left it harden for 30 min.
7. Remove the well comb from the harden gel and place the casting tray into an electrophoresis unit.
8. Add 8 µl of each PCR products in the wells starting from third well to fourteenth well.
9. Add 8 µl of deionized distilled water into the second well for negative control.
10. Take 5 µl of DNA ladder on a parafilm and mix with 1 µl of loading dye.
11. Transfer the mixture into the first well.
12. Run the electrophoresis machine at 80 V and 400 Ma for 50 mins.
13. Once the electrophoresis completes, read the agarose gel using short UV wave illumination in an Ultraviolet (UV) transilluminator to view the DNA bands.