Investigation of in Vitro Culturing Method for *Ascaris lumbricoides* Eggs in Laboratory: From Soil to Bench.

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Research note

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Abstract

Objective: *Ascaris lumbricoides* eggs are naturally available in contaminated soil and embryonated eggs are the source of Ascariasis infection to human. Investigation of *in vitro* culturing of *Ascaris* eggs in laboratory in not routinely reported in the literature. Hence, this study was carried out to investigate a simple method on *in vitro* culturing method for *Ascaris lumbricoides* eggs isolated from soil samples from an indigenous village in Malaysia.

Results: Four stages of *Ascaris lumbricoides* eggs from the first-cell stage until the fourth stage were obtained. At the embryonation stage, the larvae of *Ascaris* worm was observed indicating survival of the eggs under an acidic environment.

Introduction

More than a quarter of the world's population have higher chances of getting infection with the soil-transmitted helminths (STHs) due to the contaminated soil and it can cause several diseases [9, 12].

Ascaris lumbricoides is a nematode (roundworm) categorized under STHs, multiplies inside the gastrointestinal tract (GI tract) of human and transmitted via direct contact with the eggs presented in the contaminated soil [13]. The most prevalence of *Ascaris* infection are reported from the countries in tropical area because of warm and humid environment that could favour the transmission of the *Ascaris* infection [6].

The *Ascaris lumbricoides* female worms produces either, fertilized eggs or unfertilized eggs. The fertilized eggs are produced when female worm is inseminated by male worm; then embryonation takes place. Meanwhile, the un-inseminated female produce unfertilized eggs [15]. Fertilized eggs and unfertilized eggs both have some common and differences in terms of shape, colour, size, etc [12].

Besides, the eggs are surrounded by two layers, thicker outer shell and thinner inside the shell. The viable eggs have spaces between the inner cells of the eggs and the outer shell of the *Ascaris* eggs. The eggs are smooth, have decorticated shell and also bumpy, mamillated shell [12]. Published literature revealed high density sugar or salt solution can be used to float up the eggs/ova of helminths [4]. *Ascaris lumbricoides* eggs can grow under favourable conditions into 1-cell egg. The eggs are then advance into larva about 2 to 3 weeks where it needs a host which is human and eventually it become infective when the eggs being ingested through fecal–oral route of transmission [5]. The ova retrieved from soil evinces soil pollution and sanitation condition of the community as they may fortuitously ingested the eggs and infected with ascariasis [4].

Despite enormous reports are available on *Ascaris* cases and drug treatment, scarcely studies has been conducted on culturing the *Ascaris* eggs in laboratory conditions. Hence, the aim of this research is to optimize culturing methods of *Ascaris lumbricoides* eggs in laboratory by using sulphuric acid from various soil type and in various acidic conditions.

Methods

Study design and location

The soil sampling was conducted at Kampung Orang Asli Sungai Lalang Baru, Ulu Semenyih, Selangor, Malaysia. The study location is about 22 Kms from the university's laboratory (UniKL MESTECH, Malaysia). Some of the villagers practiced open defecation even now which resulted in soil contamination and soil transmitted helminthes (STH) propagations. The collections of soil sample were at the riverside, near the toilet area and at the pond areas.

Collection Of Soil Sample And Identification Of Soil Sample

The samples were collected by using a shovel to transfer the soil into a plastic bag prior transporting to the laboratory and used. Approximately 200–300 gram of soil were collected with a depth of 4–6 inch (optimized) in the area; common toilet area, near the riverside and near the pond area by using plastic bags and a small shovel. Upon reaching the laboratory the soil type was clearly labelled based on the location and stored in a refrigerator until used. Identification of type was carried out as previous described by Nisha et al, 2019 [10]

Overall *Ascaris* culturing process

The *Ascaris eggs* were isolated and cultured by using three different process. The primary process was to isolate the *Ascaris lumbricoides* eggs using floatation technique, next step to culture the eggs with 0.1% sulphuric acid and finally to observe the embryonation stages of the *Ascaris lumbricoides* by using a light microscope.

Isolation of *Ascaris lumbricoides*

The first method for eggs isolation from soil sample is via flotation technique. Floatation technique was used to float up the eggs at the rim of the centrifuge tube. Next, McMaster slide was used to identify and to isolate the eggs.

For this, around 3 grams of the collected soil was mixed with distilled water (15 ml) in a centrifuge tube and centrifuged for 2500 rpm and for 5 minutes. The supernatant was discarded into the sink and filled up with 15 ml of the floatation fluid which was high density salt and/or sugar solution (specific gravity; 1.28).

Upon centrifugation, the centrifuge tubes were examined for any floating eggs. The eggs were observed at the edge of the centrifuge tubes and they were collected using a disposable pipette. Later, the eggs were transferred into the McMaster chamber to be observed under the light microscope for the count and morphology.

Culturing *Ascaris lumbricoides*

Around 1 ml of 0.1% sulphuric acid was used to culture the *Ascaris lumbricoides*. Upon confirmation of the egg's morphology on the McMaster chamber, equal amount of *Ascaris* eggs were transferred into six different glass petri dishes. The ratio of *Ascaris* eggs in floatation fluid: volume of sulphuric acid was 5:1. The amount of the sulphuric acid volumes was optimized during the entire experiment.

Glass petri dishes were used instead of the normal plastic petri dishes because the sulphuric acid was highly corrosive therefore the plastic petri dishes was not recommended to due to reactivity. Next, the glass petri dishes were incubated at 37 °C for a few days and microscopic observation were carried out on daily basis up to 28 days, attributing to the life cycle of *Ascaris lumbricoides* embryonation.

Results

Isolation of STHs from soil

Two different soil types were found in the study area, namely sandy soil and loamy soil. The sandy soil near the pond area and riverside showed negative result for the presence of *Ascaris* eggs. Meanwhile, the loamy soil from all regions showed positive result and contain a few of *Ascaris* eggs. The results were shown in Table 1 below.

Table 1 Soil Types and *Ascaris* egg detection

Type of soil	Area	Result
 Loamy	Toilet	Positive for *Ascaris* eggs
 Sandy	Pond	Negative for *Ascaris* eggs
 Sandy	Riverside	Negative for *Ascaris* eggs

Culturing and observation of *Ascaris lumbricoides* eggs

From the first-cell-stage, only one developing cell could be spotted. The egg was covered with corticated layer and thick chitin shell was presence. This egg appeared approximately on day 2. Over time, the cell began to cleave into two developing cells which could be observed on day 10. On day 18, the cells were in fourth-cell-stage and the cells were still in developing process. For the final stage, a tiny larvae formed inside the egg. The outermost layer of the eggs was irregular on the surface. The outer layer of the egg

was thin proteinaceous membrane also a middle protein and chitin layer which actually gave a structural strength for the egg. Apart from that, there was presence of ascarocide layer at the innermost layer. The layer comprised of protein and unsaponifiable lipid [1] [Figure 1].

Discussions

This is the very first study to investigate culturing of *Ascaris* eggs from soil under laboratory conditions. Both loamy and sandy soil were found in the research area. The loamy soil consists of the combination of sandy particles, silt, and clay soil. Loamy soil conditions revealed high STHs egg density as previous reported by Nisha et al. [10] and it provided favourable conditions for the STHs growth. This is because the STHs eggs need warm and moist soil and the temperature should be over 18 °C to inhabit.

On the other hand, sandy soil type showed the absence of *Ascaris* eggs. This can be due to the sandy soil lack of characteristics features needed for the *Ascaris* eggs to fertilize. The sandy soil composed of high proportion of sand and a little clay inside which do not support embryonation of *Ascaris* eggs. All the soils were kept inside the refrigerator for a few days before isolation procedure technique as they tend to become dried after if it is kept for too long, the samples need to be moist for better yield. For the optimization technique, about 5 to 10 grams were kept in the incubator and incubated for 37 °C. The soil sample with viable eggs lasted up to one month in the incubator in moist conditions (distilled water was added at regular intervals to avoid dryness).

For the isolation of egg, floatation technique was used using high density floatation fluid in combination of salt/sugar solution. However, it was very difficult to get a very clear viewed of *Ascaris* eggs when observed under microscope as the soil samples contained a lot of debris and some small particles, despite sieving the soil samples few times prior to experiment.

The glass petri dishes were incubated for 37 °C and the embryonation stage were observed daily starting with the next day of the incubation. Subsequently, to avoid the dryness effect such as the solutions in the petri dishes was dried, also to maintain the humid environment for the eggs to develop, 2 to 3 ml of distilled water was added into the glass petri dishes. This technique was supported by the previous research by Bessat and Dewair in 2019[2]. Only distilled water was added to maintain moisture in the petri dishes containing the *Ascaris* eggs and the addition of sulphuric acid should be avoided since it can lead to hyper acidity affecting the developmental process.

All the stages for developmental process of *Ascaris* eggs can managed to be discovered completely on the 28th day. Each stage was observed for at least 2 days before the cells began to develop into more specialized form which at the end, the cell turned into larvae. From the previous research by Cruz et al. [5], each of the cell stage took at least 3 days to be observed except for the 3-cell stage. Temperature could be one of the factors of the viability of the eggs, from the previous study had mentioned that it could speed up the development of embryo if the temperature was higher compare to low temperature [5]. The first cell-stage of the eggs was observed between day two and day five. The morphology of the eggs observed was corticated layer, the thick chitin shell and undeveloped embryo of the eggs. The corticated

layer was a layer that surrounded the eggs. Next, for the second cell stage (day 10–15), the cells began to develop, and the cleavage could be seen in this stage. The eggs could be possibly turned into cleavage as early as day eight but this could be overlooked because the eggs were observed at the same time under microscope. Subsequently, the decorticated eggs that undergo the embryonation could be seen at day eighteen and finally the eggs had completed the embryonation period. The larvae development was seen in the last stage on day twenty-eight. The larvae was nicely captured and observed under microscope.

Apart from that, some of the eggs that were being cultivated by using sulphuric acid were incapable to develop as early as stage 2. This can be due to eggs were too fragile that the eggshells broke probably during isolating process. Next, some eggs could be immotile while cultivation due to the acidic culturing method. The amount of the sulphuric acid volumes were optimized several times till successfully *Ascaris* egg propagation were observed.

Conclusion

A simple method to culture *Ascaris* eggs isolated from contaminated soil from an indigenous village is established in this study. The floatation technique was simple and handy method to isolate the eggs from soil sample. Besides, it was found that 0.1% sulphuric acid can be used as potential acidic environment for the development of *Ascaris lumbricoides* eggs aiding in the cultivating of eggs *in vitro* condition. The finding can be used to propagate more eggs in laboratory for in vitro larvicide assessment, as the infective eggs are available naturally in environment condition.

Limitations

Limitation in this project was lack of previous research studies on this topic as references.

Abbreviations

STHs: Soil-transmitted helminths; GI tract: Gastrointestinal tract.

Declarations

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Authors' contributions

MN: Design of the study AMG: Acquisition of data. MN, AMG, FD: evaluation of data, preparation of the manuscript. All authors read and approved the final manuscript.

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Consent for publication

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Competing interests

The authors declare that they have no competing interests.

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Figures

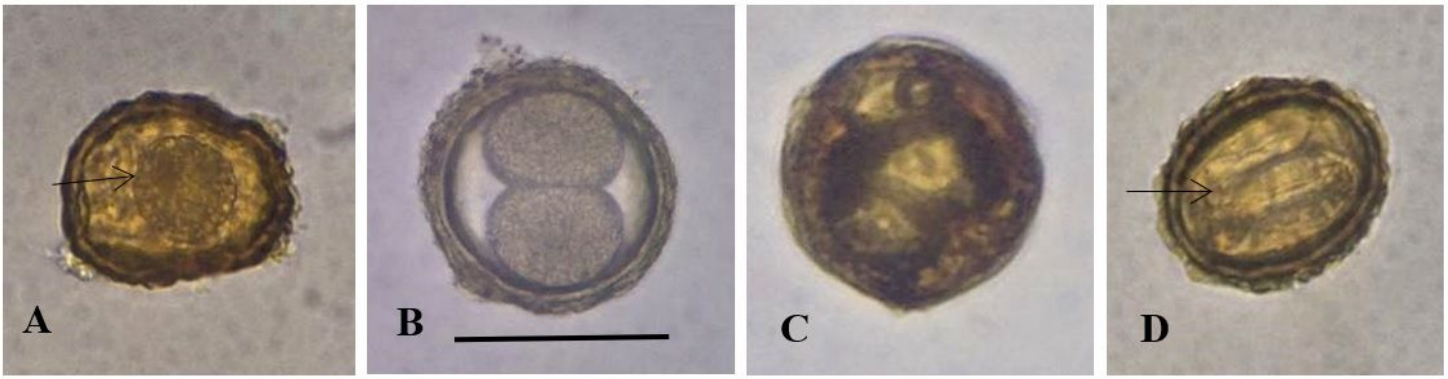

Figure 1

Photographic documentation of Ascaris eggs development. (A) first-cell stage. The egg was surrounded with a corticated layer. Arrow in (A) spotted the undeveloped unicellular egg. (B) second-cell-stage. (C) four-cell-stage. The egg undergo embryonation stage. Arrow in (D) showed larval development inside the Ascaris egg. A fully embryonated egg could be seen in (D). Bar=20 μm.