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

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Research

Keywords: Ascaris lumbricoides, Sulphuric acid, In vitro culture

DOI: https://doi.org/10.21203/rs.3.rs-93409/v1

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Abstract

Background: *Ascaris lumbricoides* is one of the four most common soil transmitted helminths (STHs), they are transmitted by direct contact with infective eggs present in the contaminated soil. Ascariasis infection causes bowel obstruction, nausea, and vomiting. Investigation of *in vitro* culturing of *Ascaris* eggs in laboratory is not routinely reported in the literature. If a simple method could be developed, it can help in testing novel drug discovery for STHs. Hence, this study was carried out to investigate on the *in vitro* culturing method for *Ascaris lumbricoides* eggs isolated from soil samples. The eggs of *Ascaris lumbricoides* were isolated from soil samples in Kampung Orang Asli Sungai Lalang Baru, an indigenous village in Malaysia.

Methods: The eggs were primarily isolated from soil using floatation technique. Next, the *Ascaris lumbricoides* eggs were cultivated using 0.1% of sulphuric acid in a glass petri dish. Then, the petri dish was incubated at 37°C in the incubator. The embryonation of the parasite was observed daily by screening samples under a light microscope. Embryonation of *Ascaris* eggs was detected in loamy soil within 2-5 days and continued to observe up to 28 days.

Results: The result showed four stages of *Ascaris lumbricoides* eggs from the first-cell stage until the embryonation stage over the time. At the embryonation stage, the larvae of *Ascaris* worm could be observed. This indicated that the eggs managed to survive and developed in the presence of an acidic environment.

Conclusion: It was found that 0.1% sulphuric acid was ideal for the development of *Ascaris lumbricoides* eggs for cultivating *in vitro* condition. In future, this method could be used to propagate the eggs for testing anthelminthic drug inhibiting the developmental stages.

Background

More than a quarter of the world’s population have higher chances of getting infection with the soil-transmitted helminths (STHs) like roundworm (*Ascaris lumbricoides*), hookworm for example *Ancylostoma duodenale*, other parasites like *Trichuris trichiura* (whipworm), *Strongyloides stercoralis* and other parasites due to the contaminated soil and it can cause several diseases [8]. Hookworm infection is spread via larval transmission, where else *Trichuris* and ascariasis are via egg ingestion [11].

*Ascaris lumbricoides* is a nematode (roundworm) and it is also referred as the large intestinal parasitic roundworm which multiplies inside the gastrointestinal tract (GI tract) of human. *Ascaris lumbricoides* is a soil transmitted helminth (STH), they are transmitted through direct contact with the eggs present in the contaminated soil [12]. Ascariasis is widespread and often reported from warm tropical and sub-tropical regions. 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 [5].
The *Ascaris* infection are found in endemic areas in developing countries from Asia and Latin America [10]. Usually, it affects children *via* contaminated food, water, or soil [6]. It is mostly reported from developing countries with poor hygiene and sanitary practices [2]. The infection can result in stunted growth as well as intellectual and cognitive loss, causes extended damage to the selected target organs like brain, liver, and urinary tract. *Ascaris lumbricoides* can also lead to bowel obstruction and volvulus in children, appendicitis, pancreatitis, anaemia due to mucosal bleeding in the upper gastrointestinal tract, asthma and also atopy [7, 13].

There are two types of eggs in *Ascaris lumbricoides* that are produce by the female worm, fertilized eggs, and unfertilized eggs. The fertilized eggs are produced when female worm is inseminated by male worm; then embryonation and development will take place. Meanwhile, the un-inseminated female produce non-embryonated eggs and for the eggs that do not undergo fertilization and it is known as unfertilized eggs. The female worm can lay about 200,000 eggs per day and the eggs are passed with faeces [14]. Fertilized eggs and unfertilized eggs both have some common and differences in terms of shape, colour, size, etc. Fertilized eggs are round oval. It measured as 45–75 µm in length while for breadth is 30–50 µm. There are stained with brown in colour for mammilated eggs while clear stained for decorticated eggs [11].

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, mammilated shell [11]. Next, fertilized eggs can float in the saturated solution of common salt. Published literature reveal that high density sugar or salt solution can be used to float up the eggs/ova of helminths [3]. This study was carried out to investigate on the *in vitro* culturing method for *Ascaris lumbricoides* eggs isolated from soil samples, at *in vitro* condition.

**Methods**

**Study design and location**

The soil sample was collected from Kampung Orang Asli Sungai Lalong Baru, Ulu Semenyih, Selangor, Malaysia. The study location is about 22 Kms from the university laboratory (UniKL MESTECH).

The village is located alongside of a river and some villagers are still using the untreated river water for their household requirements; however, majority of the villagers use clean water provided by the government. The soil sample at the riverside, near the toilet area and at the pond area were collected for the purpose of this study. The samples were collected by using a shovel to transfer the soil into a plastic bag, approximately 200–300 gram of soil were collected with a depth of 4–6 inch (optimized). Next, they were transported to the laboratory and stored and stored in a refrigerator until further studies.

**Overall Ascaris culturing process**
The soil samples were examined by using three different processes. 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*

Firstly, 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 be used 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**

Three different samples were collected from riverside, toilet area and near the pond area in Kampung Orang Asli Sungai Lalang Baru. The soils were sandy and loamy soil type 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 The result of the soil samples
### Culturing and observation of Ascaris lumbricoides eggs

Table 2 The result of cultivating *Ascaris* eggs in acidic environment from the second day up to twenty-eight days by using light microscope under 400x magnification

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

Total three soil samples that were collected from Kampung Orang Asli Sungai Lalang: loamy and sandy soil. The soil samples collected from different locations were between 200–300 grams. 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. [9] 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. These elements did not support embryonation of *Ascaris* eggs, hence *Ascaris* eggs were not be seen in a sandy type soil. The soil samples were kept inside the refrigerator for a few days before it was being used for the isolation technique. The soil samples can tend to become dried after if it is kept for too long, the samples need to fresh 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[1]. 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. [4], 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 [4]. 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, the cells began to develop, and the cleavage could be seen in this stage. The second cell stage was observed between day ten and fifteen and 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 some errors during cultivating process for instance, the
eggs were too fragile that the eggshells broke probably during isolating process. Next, the eggs could be
dead while cultivated because the ratio of acid was too high. The amount of the sulphuric acid volumes
were optimized and the other eggs managed to survive with the right proportion of sulphuric acid that
suitable for aiding in cultivating process. Furthermore, the distilled water that was mixed together with the
sulphuric acid during cultivating process could be contaminated and cause the eggs to not be able to
develop. Nonetheless, the errors were managed to overcome successfully.

**Conclusion**

The loamy soil sample from Kampung Orang Asli in Sungai Lalang Baru showed positive results of
*Ascaris* eggs. The floatation technique was a good method to isolate the eggs from the soil sample
without disturbing the fertility of the eggs. The technique was handy and simple. 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 from this study can be
used to propagate more eggs in laboratory for in vitro larvicide assessment, as the infective eggs are
available naturally in environment condition.

**Declarations**

**Funding**

No funding was involved in this project.

**Competing interests**

The authors declare that they have no competing interests.

**Consent for publication**

Written informed consent was obtained from all participants.

**Ethics approval and consent to participate**

Not applicable

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