Research Journal of Parasitology

ISSN 1816-4943

Academic Journals Inc.

www.academicjournals.com

OPEN ACCESS Research Journal of Parasitology

ISSN 1816-4943
DOI: 10.3923/jp.2020.31.37

Research Article
Additional Egg Disruption Techniques Facilitate Better DNA Yield for Species Level Identification of *Ascaris lumbricoides* and *Trichuris trichiura*

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Abstract

Background and Objective: Major species in soil transmitted helminths (STHs) infections are roundworms (*Ascaris lumbricoides*), whipworms (*Trichuris trichiura*) and hookworms (*Necator americanus* and *Ancylostoma duodenale*). Detection of these STHs is routinely done by microscopic examination from stool samples as it relatively simple and less expensive, however species level identification needs nucleic acid-based diagnostic techniques which can be challenging. Hence, this study focused on employing physical and chemical methods for the disruption of the roundworm and whipworm eggshells prior to DNA extraction. **Materials and Methods:** Primarily solid particles from stool were removed by passing through two layered damped gauze. Next, the eggs in the filtrate were concentrated by flotation in salt/sugar solutions. The eggs were counted and subjected to different disruption procedures like freeze thaw cycles, sonication, boiling and microwaving, more than one method can be used for eggs disruption. The disrupted eggs were subjected to DNA extraction using commercial kits following manufacturer's protocol. **Results:** The additional disruptions methods yielded good quality and quantity of DNA. Among the methods used, incubation of eggs in high density solutions overnight showed higher DNA yield for *Trichuris trichiura*. Boiling the eggs for a brief time followed by freeze thaw cycles depicted higher concentration of quality DNA for *Ascaris lumbricoides*. Due to the nature of the eggshells from different species-specific methods needs to be standardized to get better DNA yield. **Conclusion:** The additional methodologies recommended are highly reproducible and does not involve expensive equipment and chemicals to get higher DNA yield from STHs which are also cost effective to be performed in routine diagnostic laboratory conditions.

Key words: *Trichuris trichiura*, *Ascaris lumbricoides*, STH parasite eggs, DNA yield, rupture membrane, pretreatment eggs

Citation: Mehru Nisha, Muhammad Reduwan Sulaiman, Nurul Nadiah and Fabian Davamani, 2020. Additional egg disruption techniques facilitate better DNA yield for species level identification of *Ascaris lumbricoides* and *Trichuris trichiura*. Res. J. Parasitol., 15: 31-37.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Soil-transmitted helminths (STHs) infections are common infections worldwide that affect communities with poor sanitation facilities around the world. The round worms (*Ascaris lumbricoides*), the whip worms (*Trichuris trichiura*) and the hook worms (*Necator americanus* and *Ancylostoma duodenale*) are the major STHs species that widely cause helminth infection. According to the World Health Organization[1], over 1.5 billion people are infected with soil-transmitted helminths worldwide. The STHs eggs are transmitted by fecal-oral route and found associated with the contaminated soil which can be spread by weather conditions or various soil types.

The transmission of eggs is more rapid in tropical weather conditions and the eggs are highly fertile and facilitate easier hatching for the larvae. The signs and symptoms of STHs infection are diarrhea, abdominal pain, protein and blood loss and physical and cognitive growth retardation. Soil-transmitted helminth infections are usually treatable with oral medication with albendazole or mebendazole[2].

STHs infections are mostly reported from Southeast Asia, Southwestern China, Southern India, Sub- Saharan Africa and Central and South America[3]. In Malaysia, soil-transmitted helminths (STHs) infections are mainly reported from Orang Asli (Indigenous people) community due to their poor socioeconomic circumstance, poor sanitation and hygiene. Studies from Malaysian context from indigenous groups (Orang Asli) village in Selangor reported the prevalence in children for ascariasis, trichuriasis and hookworm infection to be 62.9%, 91.7% and 28.8%, respectively[4].

Diagnosis of STHs has always depended basically on the microscopic examination of stool samples as it is relatively simple to perform and does not require expensive laboratory equipment. The development in molecular biology in recent years, has paved way to DNA based method for STHs diagnosis. Molecular techniques have been improved over the years which had provided an advantage of rapid detection as well as accurate quantification of STHs eggs. The sensitivity of molecular techniques makes it rapid and handy to monitor the effect of treatment or control strategies[5]. There are different techniques used to extract DNA from STHs eggs from stool samples reported in the literature. In the recent past, the development of rapid diagnostic kit to extract DNA from biological samples, has replaced the earlier procedures used for DNA extraction from parasites using phenol-chloroform method which was rendered toxic and ideally not recommended to be used. However, the hard eggshells surrounding the STHs are difficult to be disrupted and hence it influenced the results with a lower yield and/or poor quality of DNA[6].

A lacuna exists in achieving quality DNA extraction from STHs eggs in a routine laboratory, using commercial kits, the results were highly varied. However, a universal technique for quantification of STHs from various samples and the assessment of species level identification remains unclear. Hence, this study aimed to develop better protocol for primary cell lysis procedures, routine equipment's used in diagnostic laboratory to rupture the membrane. Eggs of roundworm and whipworm were selected for this study based on the high prevalence data.

MATERIALS AND METHODS

Stool sample collection: Eggs were collected from soil samples positive for *Ascaris lumbricoides* and *Trichuris trichiura* from Kampung Orang Asli Sungai Lalang Baru, Ulu Semenyih, Selangor, district of Hulu Langat (3.0549°N, 101.8714°E), Malaysia in January, 2019 and stored in 70% alcohol at 4°C till use.

Concentration by floatation: Two layers of dampened gauze were used to filter the soil suspension to remove particulate matters before subjecting the samples to flotation. The filtered suspension was transferred into a test tube and filled with floatation fluid using salt solution -400 g L^{-1} NaCl or sugar solution -500 g L^{-1} sucrose with 1.2 and 1.27 specific gravity respectively. 50 mL of sugar/salt solution was used to suspend 3.0 g of soil till the rim until a convex meniscus can be seen. The eggs can be seen floating on top of the tube which was collected suspended in distilled water for enumeration.

Quantification of parasite eggs: The chambers of McMaster slides were filled with 0.15 mL of the eggs suspension after floatation and examined under 100X magnification. The number of eggs present in the grid was counted and multiplied by 50 to achieve the result of eggs per gram of feces (e.p.g.). The soil samples with the highest yield of eggs were selected for further study.

Freeze thaw cycles: In this method, the eggs in distilled water were quickly frozen immersed in liquid nitrogen for 10 min and then immediately placed in water at room temperature (~28°C) for 5 min. Alternatively, the samples were stored in -20-40°C for overnight and thawed in water at room temperature (~28°C).

Boiling and microwaving: Exposing the eggs suspension to boiling water at 100°C from 1-3 or 10 min. Microwaving the eggs suspension for 30 sec using a household microwave oven (2450 MHz).

Osmotic lysis: The eggs suspension was suspended in high density salt/sugar solution in a rotary shaker for overnight (~16 h) at 28°C, at 100 rpm. The floatation solution was used as describe above. This hypertonic solution triggers osmosis by attracting the water and causing it to move toward it, across the membrane.

Sonication: Water bath sonicator was used around 28°C and the eggs were sonicated for 30 min in pulse mode.

DNA extraction and quantification: The eggs after disruption was used for DNA extraction using commercial kits (QIAamp DNA mini stool kit Germany) as per manufacturer's protocol. Human stool or other sample types may naturally contain PCR inhibitors which was removed by Inhibit EX resin if required.

The low volume (microliter sample) spectrophotometer was used to precisely measure the wavelengths for nucleic acids and proteins. The DNA was measured as the absorbance ratios of 260/280 nm, the purity and the concentration of the DNA was estimated. The samples with/without prior lysis procedures was checked and the efficacy of DNA yield was compared. Two physical methods with highest percentage of DNA eggs lysis were chosen for DNA extraction.

RESULTS

Optimization of egg disruption methods for *Ascaris lumbricoides*: The *Ascaris* eggs were subjected to eight different treatments as additional improved/modified lysis procedures. The result indicated that the most efficient egg disruption method was by freezing at -20°C and thawing in water at room temperature and brief boiling at 100°C for 1-3 min yielded 81% of the eggs lysed. Incubation of eggs for overnight in rotary shaker in hypertonic solution yielded 78.46% lysis followed by ultra-sonication for 30 min lysed 73.6%. Freezing the samples in liquid nitrogen followed by thawing in water at room temperature yielded 72% lysis and boiling water treatment for 10 min alone resulted in 65.52% lysis, heating in a microwave oven could only disrupt 21.74%. Just freezing and thawing of eggs overnight at -40°C (10.71% lysis) and -20°C (4% lysis) had low impact on the disruption of the eggs (Table 1). However, all the treatment was able to bring certain extent of damage to the external mammillated layer (Fig. 1, 2a-e).

Optimization egg disruption methods of *Trichuris trichiura* eggs lysis: The eggs isolation and lysis procedures of *Trichuris trichiura* eggs were similar to *Ascaris lumbricoides* eggs as mentioned in section 3.1. However, it was observed that *Trichuris* eggs were more difficult to lyse due to the structural architecture of the tough egg shell. Table 2 shows the overall result for *Trichuris trichiura* eggs lysis in various treatments. The most efficient egg disruption method to lysis *Trichuris trichiura egg* was overnight rotary incubation shaker with hypertonic liquid depicted 80.65% eggs lysis, followed by freezing and brief boiling the eggs disrupted by 70.37%. Liquid nitrogen freezing resulted in 59.09% lysis, boiling water

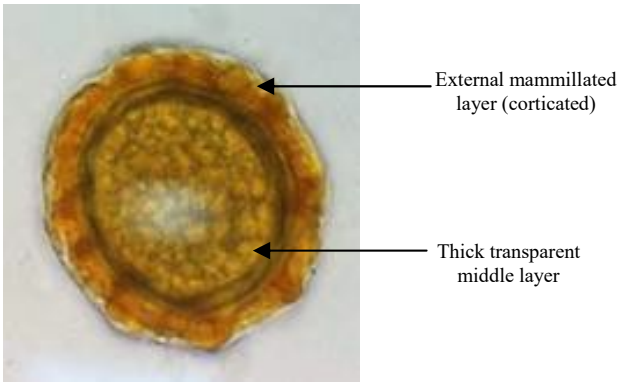

Fig. 1: Micrograph of *Ascaris lumbricoides* egg before disruption

Table 1: Efficiency of *Ascaris lumbricoides* egg disruption

Procedures	Untreated eggs count (n)	Number of eggs lysed (n)	Lysis (%)
Freezing at (-20°C) followed by boiling (100°C) 1-3 min	58	47	81.00
Overnight in hypertonic solution	65	51	78.46
Water bath sonicator for 30 min	53	39	73.60
Liquid nitrogen (-120°C) thawed in water at room temperature	64	46	72.00
Boiling water for 10 min	46	30	65.52
Heating in a microwave oven for 30 sec	23	5	21.74
Storage overnight at -40°C thawing in water at room temperature	28	3	10.71
Storage overnight at -20°C thawing in water at room temperature	21	1	4.00
Means	44.75	27.75	50.88

n: Numbers

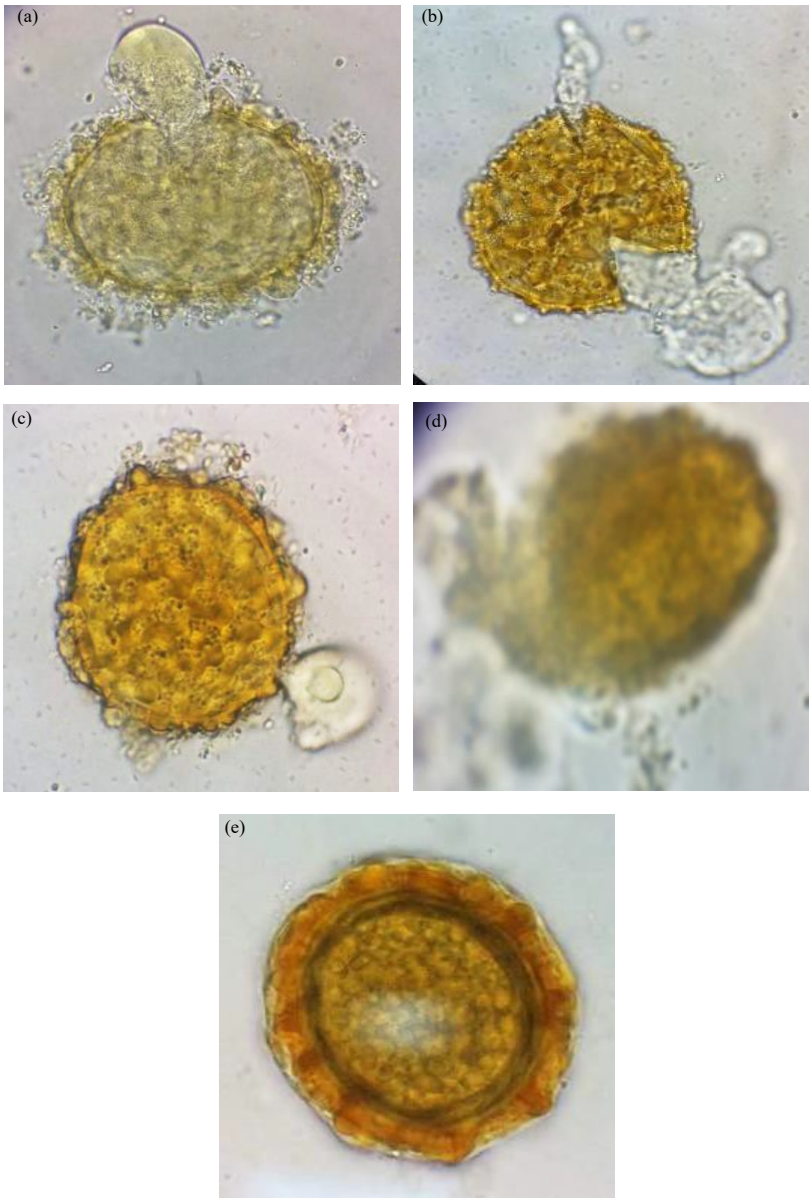

Fig. 2(a-e): Micrograph of *Ascaris lumbricoides* egg after disruption (a) Boiling following freezing/overnight incubation shaker, (b) Bath sonicator/liquid nitrogen (-120°C), (c) Immersion in boiling water, (d) Heating in a microwave oven and (e) Storage overnight at -40 to 20°C

Table 2: Comparison of various egg disruption methods for *Trichuris trichiura* eggs

Test	Initial count (n)	Number of eggs lysed (n)	Lysis (%)
Overnight incubation shaker	31	25	80.65
Boiling (100°C) followed by freezing at (-20°C)	27	19	70.37
Liquid nitrogen (-120°C)	22	13	59.09
Boiling water for 10 min	21	11	52.38
Bath sonicator for 60 min	35	15	42.86
Storage overnight at -40°C and thawing in water at room temperature	24	3	12.50
Heating in a microwave oven for 30 sec	15	1	6.67
Storage overnight at -20°C	21	0	0.00
Means	24.5	10.88	40.57

n = Numbers

Table 3: DNA quantity and quality for *Ascaris lumbricoides* and *Trichuris trichiura*

	Untreated eggs		Pre lysed eggs			
Eggs	Conc (ng µL^{-1})	Purity 260/280	Conc. (ng µL^{-1})	Purity 260/280	Modified lysis methods	Yield increased (%)
Ascaris lumbricoides	0.1	1.8	1.30	2.0	Freezing+Boiling	120
			0.74	2.0	Overnight rotary incubation shaker	64
Trichuris trichiura	0.1	1.8	1.64	1.9	Overnight rotary incubation shaker	154
			0.92	1.8	Freezing+Boiling	82

Conc: Concentration

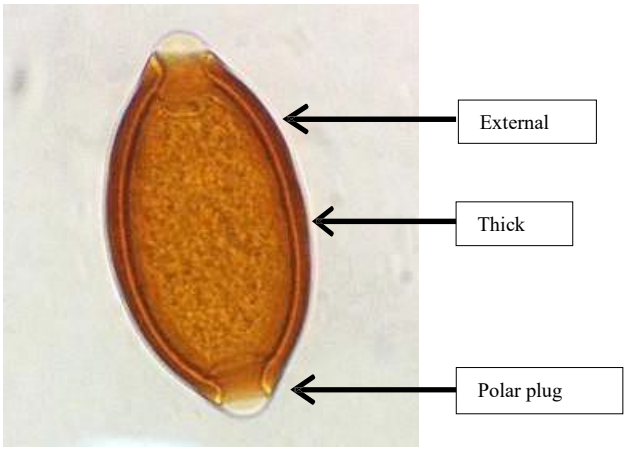

Fig. 3: Micrograph of *Trichuris trichiura* egg before disruption

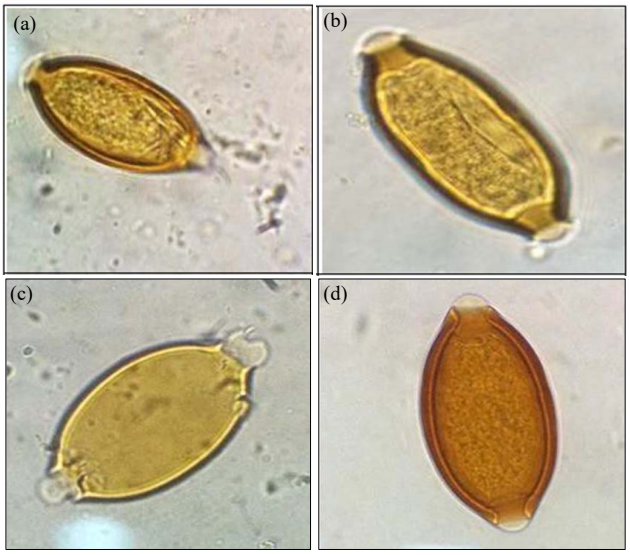

Fig. 4(a-d): Micrograph of *Trichuris trichiura* egg after disruption, (a) Boiling following freezing/ overnight incubation shaker, (b) Bath sonicator/ liquid nitrogen (-120°C)/immersion in boiling water, (c) Heating in a microwave oven/storage overnight at -40°C and (d) Storage overnight at -20°C

(10 min) resulted in 52.38% lysis, ultra-sonication (60 min) yielded 42.86% lysis, storage overnight (-40°C) lysed 12.5%, heating in microwave oven much lowered to 6.67% and freezing the eggs overnight at -20°C did not lysis at all (Table 2) (Fig. 3, 4a-d).

DNA quantification of lysed eggs: After the eggs were lysed by the former methods it was subjected to extraction of DNA. Good- quality DNA will have an A260/A280 ratio of 1.7–2.0. Lower ratios indicate more contaminants are present. DNA extraction was carried out from samples with highest eggs yield, with lysis and with untreated eggs in sample for both *Ascaris lumbricoides* and *Trichuris trichiura*. This was to quantify the *DNA yield and to* check the quality of the DNA. The control was untreated egg (Table 3).

DISCUSSION

In this study two different parasite lysis methods needed totally different procedures. Freezing the eggs suspension at -20°C and boiling at 100°C was found to be most efficient to lyse the *Ascaris lumbricoides* eggs (81.03%), the shell was susceptible for freeze thaw cycles. Whereas for *Trichuris trichiura* overnight incubation in hypertonic solution had highest egg lyse yield (80.65%). However, boiling the eggs can hydrate the shells to get denatured and denature the proteins and the heat stable DNA should not be exposed to excessive heat that needs optimum time, else it would still destroy the DNA, a fine tuning was much warranted. More studies are needed to define the fine structure and the chemical compositions of the eggs to further understanding the complete chemistry of the egg shells, so that on day better means to handle them which can be of much use in disinfection in a contaminated niche. In this study it was observed that *Ascaris* eggs lysis was better when compared to *Trichuris* eggs, this may be due to the presence of polar plugs in *Trichuris trichiura* which can give additional resistance in opening the eggs during DNA extraction process,

published literature also supports that the *Trichuris* eggs are tougher to open than other STHs[7]. Veterinarians have conducted studies on helminths, *Ostertagia* eggs it showed that using ceramic beads for lysis to improve the efficiency and better DNA yield[8], however it can incur some costing in a routine laboratory.

Despite microscopy has been considered as a gold standard to identify helminths since it can be proven without any ambiguity. However, in the recent past, molecular diagnostics report species specific identification based on the DNA, whereas with microscopic techniques species level identification needs extreme skills[9]. The advantage of nucleic based methods uses small quantity of DNA and has the ability to identify STHs eggs up to species level[10]. Based on the published data, the unique gene sequences are amplified by specific primers for PCR or quantitative PCR (qPCR). Studies have indicated that good DNA quality as a main criterion for better result for PCR based diagnosis[11], however obtaining good yield of DNA from parasite eggs remains a challenge.

The basic steps involve the isolation of parasite eggs, the crucial step is also eggs needs to be concentrated if the parasite density is very low, hence routine diagnostic laboratory may not have opportunities to do research and increase the yield by modifying the steps, usually the manufacturer's instructions are followed. Both *Ascaris lumbricoides* and *Trichuris trichiura* has hard shell that is the challenge, its mandatory for the eggs to be lysed access the DNA for isolation, whereas the DNA is armored and kept safe for the species survival, hence using physical disruption methods prior using kit may help in higher DNA yield was suggested[12], however a lacunae existed on how exactly achieve it.

The proposed methods in this study can be recommended for extended DNA extraction before using commercial kit. So the fact remains clear that untreated eggs without physical disruption treatment yielded low yield with poor quality DNA. High yield and good quality DNA is essential in molecular biology research, methods in this study satisfy both the criteria and also inexpensive and easily doable. An ardent researcher always believes that good and efficient DNA extraction protocol should yield enough DNA with high quality[13]. It is proposed that parasitologists can employ these reproducible, cost effective simple methods will be of much use in a pragmatic way for better diagnosis.

CONCLUSION

Current method of DNA extraction from helminths is performed directly using the buffers in the commercial DNA extraction kits where low yield of DNA from parasite eggs is an accepted fact by researchers. The improved methods help in higher yield as well as the quality of the DNA is improved. The methods are tested to be highly reproducible in any basic diagnostic laboratory without involving additional cost. Selection of disruption methods prior to using the kit is very specific depending on the type of eggs which is customized for *Ascaris* and *Trichuris* species.

SIGNIFICANCE STATEMENT

This study is beneficial for identification of *Ascaris* and *Trichuris* species by PCR/RT-PCR methods. The difficult part of the procedure is the DNA extraction this study helps to over come the difficulty of getting the DNA from parasite eggs. Various techniques were proposed and tested and customized for specific eggs. Its easy to identify the egg/eggs by wet mount or staining the eggs, identification up to species level by using nucleic acids our proposed methods implemented with no additional cost or special equipment's.

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