Scholarly Journal of Biological Science Vol. 7(1), pp. 1-8, January 2018
Available online http:// www.scholarly-journals.com/SJBS
ISSN 2315-6147 © 2018 Scholarly-Journals

Full Length Research Paper

Comparative Faecal Culture Techniques for Harvesting Large Quantity of Infective (Filariform) Larvae of *Strongyloides ratti*

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Accepted 5 December, 2017

Strongyloidiasis is still a major problem in the immunocompromised patients, as a result of hyperinfection syndrome and disseminated disease that is characterized with severe and sometimes fatal complications. Human strongyloidiasis research requires a large supply of infective (filariform) larvae of *Strongyloides stercoralis*. This can be achieved through in vivo maintenance of *Strongyloides stercoralis* in gerbils or in dogs which requires ethical concern and also impose possible risk of infection for the Researcher involved. However, the rodent species of *Strongyloides* are related to the human species and have been used as models for human strongyloidiasis research. This study was aimed to compare improved methods of harvesting a large quantity of infective (filariform) larvae of *Strongyloides ratti*. Baermann's Culture; the well-known and most widely used technique was compared with Modified Fecal Culture Technique (a modification of Harada Mori principle). It was observed that a total of $8.86x10^4$of pure (non-mixed) infective larvae were harvested using the MFCT compared to $4.97x10^4$of mixed stages (rhabditiform larvae, filariform larvae, and free-living adult stages) of *Strongyloides ratti* recovered by Baermann's technique within one week of continuous culture and harvest. The harvested infective (filariform) larvae from MFC technique were also observed to be more active with less contamination from fecal debris, bacteria and fungi in comparison to those harvested by Baermann's technique. It was concluded that the modified Faecal Culture Techniques (MFCT) that was employed in this study is far better than the Baermann's method for harvesting large number of filariform larvae of *Strongyloides ratti*.

Keywords: Strongyloidiasis, *Strongyloides ratti*, Modified Fecal Culture Technique (MFCT), Baermann's culture technique.

INTRODUCTION

Harvesting a desirable quantity of active filariform (iL3) larvae of *Strongyloides stercoralis* for human strongyloidiasis research is hindered by the low quantity of the parasite isolated from clinical specimen from humans (Siddiqui and Berk, 2001) and thus, the difficulty in establishing and maintaining the parasite *in vitro* or *in vivo*. To solve this problem, scientists have reported the use of its analog; the rodent species (*Strongyloides ratti,*

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Strongyloides venezuelensis) as a model for human strongyloidiasis research due to their morphological (Stanley, 1963) and phylogenetic similarities (Dorris *et al*., 2002; Mitreva *et al*., 2004) and the established protocol for continuous *in vivo* maintaining of *Strongyloides ratti* in animal model (Matsuda *et al*, 2003; Mahmuda *et al*., 2017). Faecal culture using filter paper technique was first used to harvest a large quantity of infective larvae of *S. ratti* utilizing simple and straightforward steps, as have been earlier documented (Rajasekariah *et al*., 1987). Harvesting a large quantity of infective stages of nematode parasites, especially hookworms from faecal sample using the Baermann's technique have also been reported (Baermann, 1917) but requires the use of a large volume of water which allows for the accumulation of a large quantity of fecal debris and bacteria to be harvested alongside with the larval stages, in addition to the presence of some adult stages of the parasite (Garcia, 2001).

Among the most commonly used diagnostic methods for Strongyloidiasis, Koga Agar Plate Culture (APC) has been documented to be expensive and time consuming, in addition to direct exposure of the laboratory technologist to the infective (filariform) larvae. These are the major reasons that clinical technicians do not prefer this technique as a routine procedure for the diagnosis of *S. stercoralis* (Inês *et al*., 2011). Recovery of clean and high quantity of infective filariform larvae using Baermann's techniques is difficult due to frequent contamination and high mortality rate during recovery of larvae (Inês *et al*., 2011). This necessitates continued efforts for an optional method that will allow high quantity of actively clean motile infective larvae of *Strongyloides ratti* for use in research. The main objective of this study is to compare the efficacy of the most widely used faecal culture method of Baermann's funnel with a modified faecal culture technique using filter paper in terms of high yield and quality of infective larvae of *Strongyloides ratti* intended for research purposes.

MATERIALS AND METHODS

Ethical Approval

Use of animals in this experiment was performed in line with the guidelines of the Animal Care and Use Committee, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Serdang Malaysia.

Rat Collection

Rattus norvegicus (identified based on their morphological appearance and behaviour) were caught using steel mouse traps and examined for *Strongyloides ratti* infection. All the captured wild rats were maintained at the Animal Experimental Unit, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (UPM).

Examination of Stool Samples

Stool samples were collected from the rectum of the trapped wild rats and transported at ambient temperature to the Parasitology Laboratory and processed the same day. Direct faecal smear slides were prepared and examined under a light microscope to detect helminth eggs and rhabditiform larvae.

Establishment of *Strongyloides ratti* infection model

Twenty (20) anesthetized experimental rats (6 weeks) were inoculated with approximately 2,500 infective filariform larvae (iL3) of *Strongyloides ratti* in clean water (2,500 iL3/mL) by subcutaneous injection on the dorsum of the neck as described previously (Keiser *et al*., 2008). Infection status was examined for a period of ten (10) weeks. Fecal pellets were collected (per rectum) daily from 7 days after inoculation of the rats and were examined for worm-egg count (EPG) using Modified McMaster technique and cultured (MFCT) thereafter for larval detection and harvest according to the procedure of Kotze *et al*., (2004).

Modified McMaster worm-egg counting technique

Microscopic egg-count was carried out using Modified McMaster method to determine the number of eggs per gram (EPG) of feces. Briefly, three grams (3 g) of fecal sample collected from infected rats was thoroughly mixed with 42 mL of tap water and was strained through a wire mesh tea strainer. Three (3) mL of the supernatant (filtrate) was transferred to a centrifuge test tube and was centrifuged at 2,000 rpm for 3 minutes, after which the sediment was obtained from decanting the supernatant. Ten (10 mL) of floatation fluid (Saturated NaCl solution) was added to the sediment in the test tube and was inverted 6 times and shaken to ensure proper mixing. Using a pipette, the mixture was used to fill the two chambers of the McMaster slide and was examined under low magnification (10 X). Eggs within the ruled margins of the McMaster slide were counted and multiplied by 100 as a multiplication factor to obtain the number of eggs per gram of faeces (Taylor *et al*., 2007).

Modified Fecal Culture Technique (MFCT)

Modified Faecal Culture Technique (MFCT) coined from Harada Mori principle for coprocultures was performed. Faecal samples (10 g) were collected from the rats in a 50 mL centrifuge tube each. Twenty (20) millilitre of sterile water (contained 100 IU/mL Penicillin, 100 µg/mL

Streptomycin, and 2.5 µg/mL Fungizone) was added into the tube and mixed well using applicator stick until completely homogenous. A few drops of the solution were then examined under the microscope for the confirmation of eggs and rhabditiform larvae. Whatmann No. 1 filter paper pairs were prepared and each pair was laid on top of a clean tissue absorbent paper inside the fume cabinet. Drops of the solution were then spread on the surface of each of the pairs of filter papers and then let it dry for 10 minutes inside the fume cabinet (Plate 1A). This was to allow the tissue absorbent paper to absorb all the excess water. Each of the pairs of filter papers were then transferred inside clean plastic petri dishes (Plate 1B). A few drops of sterile water (contained 100 IU/mL Penicillin, 100 µg/mL Streptomycin, and 2.5 µg/mL Fungizone) were added onto the outer cycle of the filter paper (but not to flood the filter paper). All the petri dishes were then covered, well labelled and sealed with parafilm (Plate 1C). This was to prevent the infective larvae from crawling out of the petri dishes. All the petri dishes were then incubated inside a 28°C incubator (Labwit Incubators, China) for two days on an inverted position (Plate 1D). This was to allow the formation of condensed water on the lid cover of the petri dish.

After two days, a few drops of the condensed water formed on the lid cover were examined under stereo-microscope for the presence of infective larvae. All the condensed water from the petri dishes were collected and pooled inside a 15 mL centrifuge tube. This was done by adding 1 mL of sterile water (contained 100 IU/mL Penicillin, 100 µg/mL Streptomycin, 2.5 µg/mL Fungizone) to the cover lid and gently washes the surface of the cover lid and then all the solution from all cover lids were transferred into two clean 15 mL centrifuge tubes (each contained 5 mL of solution). The centrifuge tubes were spun at 500 rpm for 10 min. Four (4) mL of the supernatant was gently discarded from each tube. The remaining 1 mL was then examined for the presence of infective larvae using stereo microscope (Olympus, Japan). A new 4 mL of sterile water (contained 100 IU/mL Penicillin, 100 µg/mL Streptomycin, and 2.5 µg/mL Fungizone) was gently added into the tubes. The centrifuge tubes were spun at 500 rpm for 10 minutes, and then 4 mL of the supernatant was gently discarded from each tube. The remaining 1 mL was then examined for the presence of infective larvae using stereo microscope as above.

Baermann's Culture Technique

Baermann's cultures were organized by mixing activated charcoal (Merck, Germany) with *Strongyloides ratti* infected faeces collected from the rats. The mixed feces were cultured in covered petri dishes at room temperature for 48 hr after which it was transferred to a gauge and incubated in a plugged glass funnel apparatus containing warm (37°C) water for 30 min. The filariform larvae (iL3) were allowed to migrate out of the fecal mixture, into the water and were collected at the bottom of the funnel as previously described (Reiss *et al*., 2007). The structures (total length, width, length of oesophagus and number of worm eggs in the uterus) of the different stages (parasitic females, free-living males and females and filariform larvae) of *Strongyloides ratti* identified were analyzed using Image Analysis Software (Nikon Corporation, Japan) for comparison.

Larval Count

The estimation of the total number of larvae (iL3) harvested was done by counting the number of larvae in 20 µL multiplied by 50 (for 1mL) and multiplied by the total volume in milliliter (mLs) after final washing according to the procedure of Van Bezooijen (2006), and the larvae were then aliquotted in cryovials (Nolan and Schad, 1992) and stored at -80°C until required.

Maintenance of *Strongyloides ratti* infection

The infection in the experimental rats was maintained by oral prednisolone administration at 0.25 mg/kg body weight per day for a maximum of four (4) weeks, after the infection status is examined to be low (Chronic infection) as previously described (Mahmuda *et al*., 2017).This was to induce an experimental immunosuppression in the rats in order to maintain the infection.

Experimental design and Drug administration

At the end of the ten (10) weeks experiment above, the twenty (20) rats were divided into two groups of ten (10) rats each. Animals (rats) in the first group (experiment 1) were treated by oral administration of prednisolone solution with 0.25 mg/kg body weight daily for four (4) weeks. In the second group (experiment 2), rats were treated by oral administration of prednisolone with 0.35 mg/kg body weight daily for four (4) weeks. The first and second experiments were performed concurrently. Daily worm-egg counts were recorded, after which five (5) rats from each group were humanely sacrificed. The intestines from all sacrificed infected rats were considered and processed for the presence of adult and parasitic females.

Statistical Analysis

Data of worm-egg count were expressed as the means (Mean ± SD) of independent experiments and were analyzed using SPSS, version 20.0. Results were compared between groups using one-way analysis of variance (ANOVA), and significant differences ($P < 0.05$) were determined using the Duncan' multiple range test.

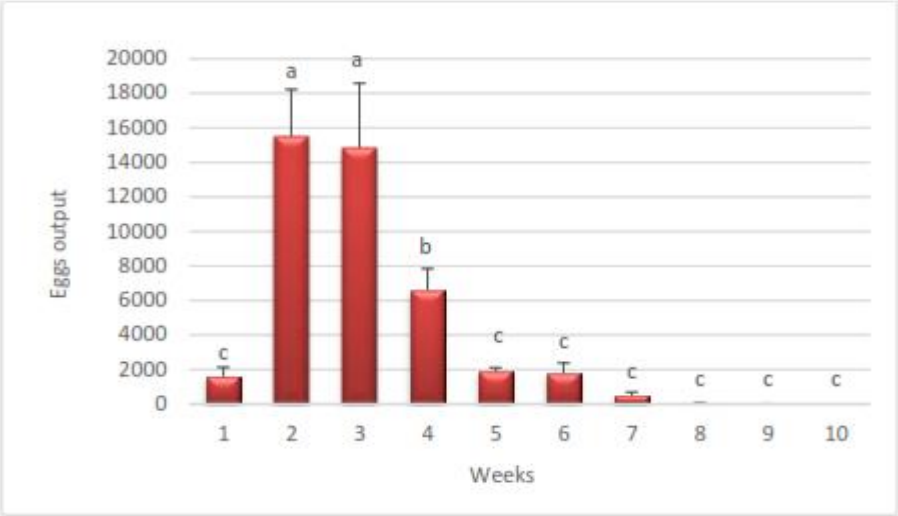

Figure: 1: Eggs output of chronic infection of rats with *S. ratti*. Means with same letters are not significantly different (P< 0.05).

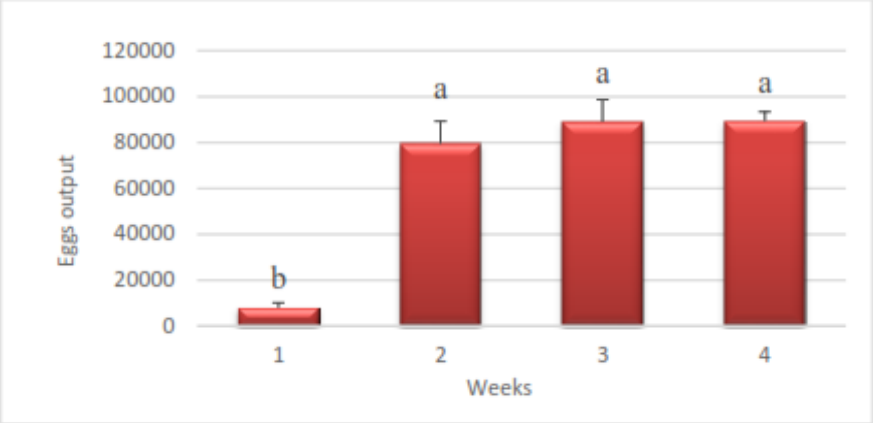

Figure: 2: Eggs output of *S. ratti* after four weeks treatment with 0.25 mg/kg Prednisolone. Means with same letters are not significantly different (P< 0.05).

Part of the data were subjected to descriptive type of statistical analysis as expressed in table and bar charts.

RESULTS

Chronic Strongyloides infection in a rat model

An infection that was initiated with 2,500 infective filariform larvae (iL3) became patent within 6 days post-infection. The daily mean egg output reached a peak at 15,510 egg per gram (EPG) of feces two weeks post-infection, and then declined gradually to zero by week 10 (Figure:1).

Effect of corticosteroids on strongyloidiasis

In the first experiment, ten (10) rats with chronic infection (zero EPG) were treated daily with prednisolone at 0.25 mg/kg body weight for four (4) weeks. The daily mean egg output increased from zero reaching a peak at 89,340 EPG in the fourth week (Figure: 2). Thereafter, 5 rats from the group were sacrificed. However, approximately 5,000 adult worms were collected from the small intestine. A considerable number of parasitic females were isolated from the small intestines.

In the second experiment, ten (10) rats with chronic infection (zero EPG) were treated daily with an increased dose of prednisolone at 0.35 mg/kg body weight for four

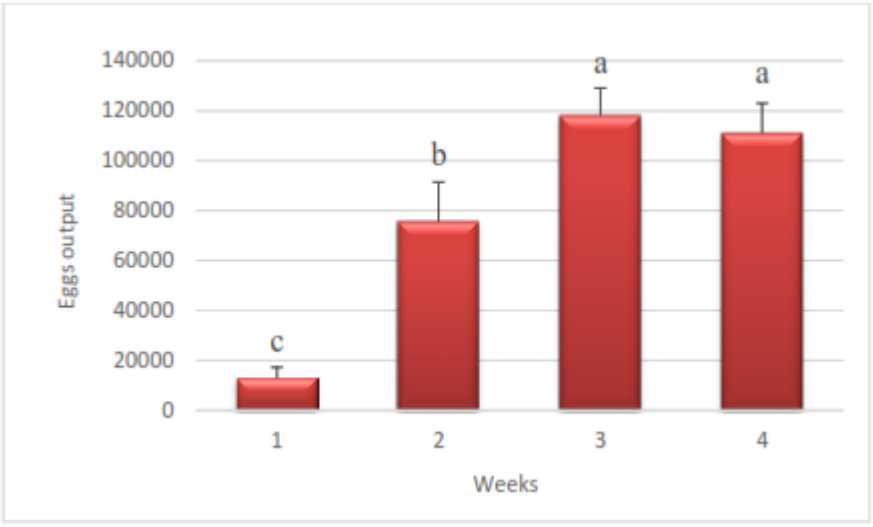

Figure: 3: Eggs output of *S. ratti* after four weeks treatment with 0.35 mg/kg Prednisolone. Means with same letters are not significantly different (P< 0.05).

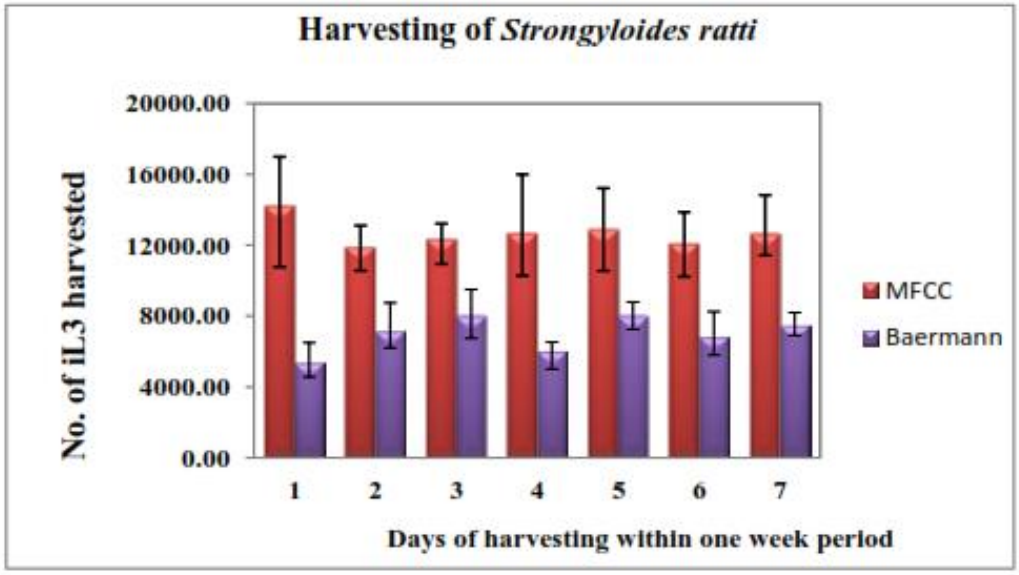

Figure 4: Comparative Harvest of infective (filariform) larvae of *Strongyloidesratti* over one week period using Modified Faecal Culture and Baermann's Culture Techniques.

MFCC = only filariform (iL3) larvae were harvested (8.86 x 10^4 iL3)
Baermann = mixed stages (4.97 x 10^4iL3 larvae and adults)

(4) weeks. The daily mean egg output increased from zero reaching a peak (117,976 EPG) three weeks later and then dropped insignificantly to 110,960 EPG (P < 0.05) at the fourth week (Figure:3). Similarly, a considerable number of parasitic females were isolated from the small intestines.

A total of 8.86 x 104 iL3 larvae were harvested using MFCT compared to 4.97 x 104 mixed stages of *Strongyloides ratti* recovered by the Baermann's technique over one week of continuous harvesting (Figure 4). The harvested infected larvae from the MFC technique were more motile and contained less contamination from faecal debris, bacteria and fungus when compared to the Baermann's technique (Table 1). Comparative measurements of worm structures of the *Strongyloides ratti* stages was performed with recorded

Table 1: Comparison of Baermann and Modified faecal filtration culture techniques in relation to preparation and incubation time, yield, mortality rate of the infective stages and purity.

Items	Baermann Culture(Room temperature)		MFCT (Incubation at 28^{O}C)	
Time required	Preparation	Incubation	Preparation	Incubation
	48 h	2-4 h.	30 min.	48 h
Isolated stages	Adults and filariform larvae		Filariform larvae (iL3)	
Yield (in one week continuous harvesting)	4.97 x 10^4 mixed stages		8.86 x 10^4 iL3 larvae	
Mortality rate	40%		1%	
Faecal debris/Microbial tamination	High		Low	

Plate1: Stages involved in performing Modified Faecal Culture Technique using filter paper

similarities and minor differences observed (Table 2).

DISCUSSION

Considering the difficulty associated with in vivo maintenance of experimental infection of *Strongyloides stercoralis* for adequate isolation of a significant number of infective (filariform) larvae, this study reported that it is much easier to keep rats infected with *Strongyloides ratti* than maintaining dogs as previously suggested (Gonçalves *et al*., 2007) or monkeys infected with *Strongyloides stercoralis*. Similar studies have previously reported use of corticosteroids for experimental immuno-suppression of rats infected with *Strongyloides ratti* (Basile *et al*., 2010) and *Strongyloides venezuelensis*

Table 2: Comparative measurements of *S. ratti* stages in the present and previous studies.

	Comparative measurements					
	Length (mm)	**Width (mm)**	**Length of Oesophagus (mm)**	**Mouth to vulva (mm)**	**Anus to tail (µ)**	**No. of eggs in uteri**
Parasitic female						
Sandground (1925)	1.85-3.03	-	0.33-0.78	1.27-1.73	32-50	-
Little (1966)	2.1-3.1	30-38	0.73-0.76	1.4-1.9	45-65	6-8
Present study	2.8-3.2	0.075	0.53-0.79	1.5-1.93	38-55	5-8
Free-living male						
Sandground (1925)	0.82	0.038	-	-	-	-
Little (1966)	-	-	-	-	-	-
Present study	0.92-1.0	0.029	-	-	-	-
Free-living female						
Sandground (1925)	1.12-1.2	0.04	-	-	-	-
Little (1966)	-	-	-	-	-	-
Present study	0.98-1.5	0.06	-	-	-	-
Filariform larvae						
Sandground (1925)	0.56-0.73	0.17-0.21	-	-	-	-
Little (1966)	-	-	-	-	-	-
Present study	0.62-0.78	0.19-0.3	-	-	-	-

(Tefé-Silva *et al*., 2008; Machado *et al*. 2011). Oral administered prednisolone efficiently induced immunosuppression and resulted in an increased number of eggs in the feces (EPG) and also an increased in larval yield. This finding is also supported by a previous report (Ohnishi *et al*., 2004).

Baermann and Koga Agar Plate Culture methods are the two most commonly used methods for diagnosis and research application, especially in relation to human strongyloidiasis. Both methods are excellent diagnostic tools in term of reliability, reproducibility and accuracy in the identification of *S. stercoralis* from patient stool specimen (Baermann, 1917; Koga *et al*., 1990, 1991). However, APC is time consuming to perform, involves high costs and direct exposure of the Laboratory technician to infective larvae (Arakaki *et al*., 1990). These are the major reasons that clinical technicians do not prefer this technique for the routine detection of *S. stercoralis* (Inês *et al*., 2011). The recovery of high quality and quantity of infective filariform larvae (iL3) using these techniques is difficult due to partial contamination of the agar and the presence of bacteria, also due to the high mortality rate during recovery of larvae (Inês *et al*., 2011).

This study has successfully established the continuous *in vivo* maintaining of *Strongyloides ratti* in rats (Wistar). In terms of harvesting a large quantity of *S. ratti* infective (filariform) larvae for research purpose, the study was able to establish that the MFC technique yielded more active infective larvae compared to Baermann's technique. The filariform larvae harvested from MFFC were much cleaner and more motile compared to the (iL3) larvae harvested using the Baermann's technique. The MFC technique has supplied us with a large number of *S. ratti* infective larvae needed for the production of somatic and excretory-secretory antigen. During the incubation period of 2 days at 28°C, formations of condensed water droplets on the edges of the lid cover of the petri dishes allowed the more active infective larvae to migrate and concentratein thewater droplets, whilethe slow migrating adults moved only slowly within the tissue paper in the petri dish. All the condensed water droplets were collected and pooled by adding sterile water (contained 50 IU/mL Penicillin, 50µg/mL Streptomycin, 2.5µg/mL Fungizone). This allowed for easy separation of infective (filariform) larvae from the adults and resulted to less contamination from fecal debris, fungi and bacteria.

The use of the large volume of water required in Baermann's method during filtration results in accumulation of a large amount of debris, including bacteria, fungiand adults worms, making harvesting and isolating the infective (iL3) larvae more difficult. The MFCT uses 50IU/mL Penicillin, 50µg/mL Streptomycin, 2.5µg/mL Fungizone and less amount of water for culturing and harvesting the infective (filariform) larvae which results into less contamination from bacteria and fungi. Nevertheless, the bacterial contamination may still be present, but much less compared to Baermann's technique. The Baermann's technique yielded both adults and larvae concurrently during harvesting, which make separation between the stages more difficult. This study has shown that MFC technique is a better technique to use for the purpose of harvesting a large quantity of only infective (filariform) larvae of *Strongyloides ratti* for research purposes. The observed differences and similarities as were compared (Little, 1966; Sandground, 1925) in the measurements of structures of the different stages of *Strongyloides ratti* (parasitic females, free-living males and females and filariform larvae) maybe due to

type of image analysis software used. This further highlights the significance of the morphological characterization process.

CONCLUSION

This study have concluded that the modified faecal culture technique (MFCT) employed is far more effective than the conventional Baermann's method for harvesting large quantity of filariform larvae of *Strongyloides ratti* which were free from many contaminants, especially for research purpose. The possibility of using this method for culture of other nematode parasites can as well be determined.

Conflict of interest

The Authors declare that there is no conflict of interest regarding the publication of this paper

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