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博 士 論 文

Re-evaluation of the formalin-ether sedimentation method for the improvement of recovery efficiency of parasite eggs

(虫卵回収率の向上を目的としたホルマリン・エーテル法の再評価)

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Re-evaluation of the formalin-ether sedimentation method for the improvement of recovery efficiency of parasite eggs

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ABSTRACT

The formalin–ether sedimentation (FES) method is a reliable method of fecal examination used by many researchers. We examined several factors of this method to re-evaluate its efficiency: (i) pre-treatment of feces; (ii) filtration of fecal suspensions; (iii) test-tube material; (iv) efficacy of organic solvents substituted for ether. The method was based on the original procedures of the formalin–ether sedimentation method. The egg count was represented by the number of eggs detected per 100 μg in the sediment. Pre-treatment of feces with formalin (pH 7) increased egg detection remarkably compared with original procedures. Replacing it with three layers of gauze dramatically reduced the sediment in the final product, and led to an increase in the number of eggs detected. The use of polypropylene test tubes instead of glass test tubes also increased egg detection. Several organic solvents were tried in place of ether, but none of them produced better results. Based on these findings, we proposed a modified procedure for the formalin–ether sedimentation method. Next, we compared the prevalence of infection and number of egg recovered from the original FES and the modified FES methods by using 112 fecal samples collected from children in an endemic area of parasites in Nepal. The reason for this result could be related to the intensity of parasitic infection in the fecal sample. Feces collected from Nepal had many parasite eggs, and these fecal samples barely displayed false-negative results even if a low sensitivity method was used. When the mean number of egg recovered between the two methods was compared, all values obtained from the respective parasites of *Vampirolepis nana*, hookworm, *T. trichiura*, and *A. lumbricoides* were superior in the modified FES. This result suggested that the modified FES is effective in areas of low-intensity parasitic infection.

INTRODUCTION

Various methods can be used for parasite detection in fecal samples: direct smear, flotation and centrifugal sedimentation. For the detection of helminth eggs or protozoan cysts, the most commonly used concentration-based method is the formalin-ether sedimentation (FES) method, which is one of the centrifugal sedimentation method. This method was reported by Ritchie in 1948.¹ He found FES method superior to the direct smear method and other centrifugal sedimentation methods in the recovery of helminth eggs and protozoan cysts. Since then, FES method has undergone various modifications²⁻⁴, and the one now being used is regarded as one of the most reliable methods for the detection of helminth eggs and/or protozoan cysts in fecal samples.⁵

Several problems were noted in original FES method. For example, Ritchie's report¹ did not mention precise name of parasite despite the detailed descriptions of FES procedures. Similar problems were in the findings reported by Young *et al.* in 1979.² They described the suitability of ethyl acetate as a substitute for ether, but mentioned nothing about the amount of sediment because of a restricted focus on egg count. Parija *et al.*³, who used acetone as a substitute for ether, have also come up with similar conclusions. Kightlinger and Kightlinger⁶ mentioned about the usefulness of detergents in the method modified by Young *et al.*² but without mention about the egg recovery efficiency in relation to the use of different types of test-tube or the number of gauze layers used for filtration. Available reports on FES method modifications have examined only the procedural part and the expression of results was not considered.^{2, 3} Reports discussing FES method in a comprehensive manner have not available.

According to an epidemiological survey on intestinal parasites conducted in Indonesia by Uga *et al.*⁷, positive samples contained less number of eggs (only several eggs). This appeared to be attributed to a low intensity of parasitic infection in the survey area. However, Pegelow *et al.*⁸ and Toma *et al.*⁹ four to five years earlier have

reported a high prevalence of parasitic infection in that area. These differences might have been associated with the intensity of the parasitic infection. Sensitivity of FES also must have been associated with these results. Other reports also have not indicated high recovery efficiency of FES. Utzinger *et al.*¹⁰ reported higher sensitivity of Kato-Katz direct smear method than that of the FES in detecting hookworm eggs. Hong *et al.*¹¹ also showed similar results for *Clonorchis sinensis* eggs.

Japanese dietary habits have become increasingly diversified and meat-oriented over the past 50 years, similar to Europe and the United States.¹² According to recent data, intake of animal fat and protein in Japanese society has increased by approximately tenfold and fourfold, respectively compared to late-1950s.¹² have also been observed in many countries worldwide. Therefore, change in food constituent seems to have affected fecal characteristics and lowered the sensitivity of FES. In spite of this, a full-scale re-examination of FES method developed in the past has not been re-assessed for the detection of parasites in fecal samples in current conditions. We, therefore, evaluated the parasite egg recovery efficiency of the original FES method devised by Ritchie¹ by examining different aspects/steps of the procedure comparing with modified FES using fecal samples collected in parasite endemic area.

MATERIALS AND METHODS

Examination of the basic conditions in the respective processes of FES

(i) Preparation of fecal samples

For accurate measurement of the efficiency of egg recovery, specified (known) number of *Diphylllobothrium latum* eggs were mixed with dog feces and suspended with specific volume of 10% formalin solution. This fecal suspension (standard suspension) was examined by direct smear method using 20 μ L of suspension, and the number of eggs was counted. The volume of suspension examined was such that produce 0.5 g of

sediment when centrifuged at $700 \times g$ for 10 min at room temperature. For preliminary study, instead of dog feces, gorilla feces containing hookworm egg was used. For comparative study of parasite egg detection efficiency of original FES and modified FES methods, one fecal sample collected from an endemic area that contained the fertilized eggs of *Ascaris lumbricoides* and hookworm eggs was used. In addition, we also used 112 fecal samples from parasitic endemic areas to compare the egg recovery of the original FES to the modified FES methods. These feces were collected school children (aged 5-16 years) in 2013 at a public elementary school in Nepal.

(ii) *Re-examination of FES procedure*

The original FES procedures of Ritchie¹ and Price¹³ were used. Briefly, 8 mL of 10% formalin solution was added to 0.5 g sediment of standard suspension. After 30 min, this suspension was filtered with one layer of gauze and centrifuged at $700 \times g$ for 2 min at room temperature. The sediment was then diluted with a 10% formalin solution to make a total volume of 6 mL, and to this, 2 mL of ether was added. The test tube was sealed, shaken vigorously for 30 s, and centrifuged again at $700 \times g$ for 2 min at room temperature. After centrifugation, the supernatant (consisted of three layers: ether, feces and formalin) was discarded and the remaining sediment was adjusted to 200 μ L in total with the addition of few drops of 10% formalin solution. Twenty microliters of sediment (concentrated sample) was used for observation under a light microscope. Of the original FES method, we selected four components (pre-treatment of feces, filtration, test-tube material and substitutes for ether) and examined the optimal conditions for these components. We employed these components into the original FES procedures in this study. Then, the original FES procedure was carried out, with modifications made to each of the four components using a standard suspension. The final amount of sediment produced from the standard suspension and the number of parasitic eggs per 100 μ g of sediment was recorded.

(a) *Pre-treatment of feces*: Several adjustments in the 10% formalin solution used to suspend the feces were investigated. These were, addition of (i) 10% formalin solution containing 0.001% gelatin, (ii) 10% formalin solution containing 0.001%, 0.05%, and 0.01% Tween 80, (iii) 10% formalin solution adjusted to pH 7 or 10 and (iv) 10% formalin/feces suspension treated thrice with 20-s sonication.

(b) *Filtration*: One-to-four layers of gauze (type-I gauze in compliance with the Japanese Pharmacopeia) were used for the filtration of the fecal suspension.

(c) *Test-tube material*: To examine the effect of test tube material (type) on the egg recovery efficiency, different tubes were used. These included glass tubes (Maruemu, Osaka, Japan), silicon-treated glass tubes (Sigma–Aldrich, St. Louis, MO, USA), polyethylene terephthalate tubes (PET; Asone, Osaka, Japan), and polypropylene tubes (IWAKI, Tokyo, Japan).

(d) *Ether substitutes*: Ether has been used in the original FES method. The usefulness of alternative solvents such as ethyl acetate, acetone, alcohol-ether mixture (1:1), xylene, toluene, and methyl ethyl ketone (Wako, Osaka, Japan) were examined.

Comparison of egg concentration rate by FES procedures

Once the original FES and modified FES methods were performed, the egg concentration rate by these procedures was compared. Specifically, the total number of eggs supplemented in the gauze, ether, feces, formalin, and sediment was observed. Amount of sediment and observation time was recorded during both FES methods. To ensure the accuracy of data collected during the procedure, the procedures were performed by the same individual.

Comparison of egg detection from fecal samples

The fecal samples collected from school children in parasite endemic area (n=112) were examined by both original FES and modified FES methods. The prevalence, types of parasites detected and the intensity of the infection were compared based on the

results of these two methods/procedures. The intensity of infection was determined by counting and comparing the number of eggs detected in 20 µg of the sediment obtained by two FES methods/procedures.

RESULTS

Examination of the basic conditions in the respective processes of FES

(i) Re-examination of FES procedure

(a) *Pre-treatment of feces*: Fig. 1 shows the effect of various pre-treatments of fecal suspension on the recovery of sediment and eggs. The bar diagram shows the total amount of sediment whereas the line displays the number of eggs detected in 100 µg of sediment.

Addition of gelatin or different concentrations of Tween 80 to the fecal suspension did not improve the recovery of sediment and eggs. Pre-treatments such as sonication or adjustment of pH to 10 did not improve the recovery either. In contrast to these findings, adjustment of the fecal suspension to pH 7 dramatically reduced the amount of sediment, which led to a remarkable increase in the egg count compared with the original procedure (i.e., 33 eggs/100 µg of sediment was the result of original FES method whereas the number of eggs detected increased to 107/100 µg of sediment when pH was adjusted to 7; $p < 0.05$).

(b) *Filtration*: Fig. 2 shows the effect of filtration on the recovery of sediment and eggs. In this examination, filtration was done using one to four layers of gauze. As the layers of gauze increased the amount of sediment decreased. The maximum egg count per 100 µg of sediment was obtained with the use of three layers of gauze. For instance, the egg detection rate in 100 µg of sediment was 73 in the sediment obtained with the use of one layer gauze (used for filtration) while it was 125 with two layers and 147 with three layers and these increase in egg detection was significant ($p < 0.05$). The egg recovery

was reduced to 115 when sample was filtered through four layers of gauze despite the smaller amount of sediment obtained. Therefore, we concluded that filtration of samples using three layers of gauze was optimal.

(c) *Test-tube material (type)*: Of the different types of tubes used in the procedures the recovery of eggs in the sediment was significantly higher when polypropylene tubes were used instead of glass tubes ($p<0.05$) (Fig. 3).

(d) *Ether substitutes*: Ethyl acetate, acetone, alcohol-ether mixture, xylene, toluene and methyl ethyl ketone were examined as possible substitutes for ether in FES method (Table 1). Of these, ether proved to be the better in obtaining a minimum amount of sediment (the final product) with a maximum egg recovery, but the difference was not significant ($p>0.05$).

Comparison of egg concentration rate during FES

In this experiment, human feces with 15,525 fertilized eggs of *A. lumbricoides* and 1,225 hookworm eggs was used. The original FES procedure performed using this fecal sample revealed few *A. lumbricoides* and hookworm eggs in ether and formalin layers while most of the eggs were trapped in gauze, fecal layers and sediment. Of the total, 31% of *A. lumbricoides* eggs and 23% of hookworm eggs were found in the sediment. But in the case of modified FES procedure with three layers of gauze, more eggs were found trapped in the gauze and only few eggs in fecal layer. When compared with the original method, the modified FES method yielded less amount of sediment (by 14%) and this made easy in microscopic observation. The number of eggs in the sediment was much higher compared with the original FES method (Fig. 4). Furthermore, the time taken for examination was also decreased (7% decrease compared with the original FES procedure).

Comparison of egg recovery within feces from endemic areas

The comparative results of parasite egg detection by original FES and the modified FES procedures are shown in Table-2. In this experiment, eggs of four species parasites species namely, *Vampirolepis nana*, hookworm, *Trichuris trichiura* and *A. lumbricoides* were detected. The mean number of eggs of these parasites detected by original FES procedure was 197, 22, 19, and 142, respectively whereas it was 300, 29, 22 and 222, respectively by modified FES procedure. The total number of egg detected, irrespective of species, was much higher by modified FES procedure.

Modified FES procedure was relatively more sensitive compared with the original procedure. Of the total 112 fecal samples examined by both original and modified FES procedures, the modified procedure detected parasite eggs in 28% (31/112) stool samples whereas the original procedure detected only in 24% (27/112) of samples. However, this improvement was not significant ($p>0.05$). Interestingly, all four stool samples negative by original method but positive by modified method had *T. trichiura* eggs and the number of eggs in these four samples was 9, 9, 1 and 89.

DISCUSSION

Various methods can be used for fecal examination including those centrifugal floatation and sedimentation. In this study, we chose FES method for re-examination despite it is known to detect both helminth eggs and protozoan cysts without causing much morphological change compared with other methods such as flotation and/or Kato–Katz methods. To evaluate the FES method, we considered four factors in the procedure focusing on the amount of sediment produced and the number of egg recovered.

Approximately 200 μ L of sediment was obtained by original FES procedure out of which 20 μ L was used for wet mount microscopic examination. In this case, the relative egg count was low despite large number of eggs present in the sample. This result

suggested that more amount of sediment reduces the microscopic detection of eggs in the sediment. It was assumed that the reduction in the amount of fecal sediment should be the key factor in preventing false-negative results. This assumption corresponds to the suggestions made by Price¹³, who recommended mounting of almost the entire amount of sediment from a tube directly onto a slide to eliminate the loss of eggs and thereby avoid false-negative results. When the pH level of formalin solution was adjusted to 7.0, the efficiency of egg recovery was increased compared to original FES methods. Our result was consistent with that of Richie *et al.*¹⁴, who demonstrated the effect of pH in egg recovery. Richie *et al.*¹⁴ and Oshima *et al.*¹⁵, however, stated that the optimal pH level varied according to parasite species. The preliminary study targeted only hookworm eggs so we cannot draw conclusions as to the effect of pH on recovery efficiency in other helminths. In general, gauze is used for filtration of fecal suspensions in FES procedure. Previously, filtration of fecal suspensions with one layer of gauze was recommended by Takahashi *et al.*¹⁶ and Nakanishi *et al.*¹⁷ whereas by Ritchie¹ and Young *et al.*² recommended two layers. However, in this study, use of three layers of gauze proved to be the most effective for parasite egg recovery. Gauze is expected to trap most of fecal particles larger than eggs and therefore makes easy for microscopic observation. Pamba and Mulega¹⁸ used metallic filters for filtration in FES procedure. Unlike gauze, metal trap does not absorb fecal suspension. However, it also traps parasites eggs together with fecal particles including nematode larvae. Therefore, it seems that a metallic filter is not a suitable for filtration of fecal suspension for FES procedure. Findings of present study suggested that use three layers of gauze for filtering fecal suspension is more effective than one or two layers in reducing the amount of sediment and thereby in parasite egg recovery. However, further study is required to determine whether the similar results can be obtained from feces containing larger sized parasites eggs such as eggs of *Fasciola hepatica* or eggs with higher

specific gravity (such as trematode eggs).

Price¹³ pointed out that feces adhere to the glass surfaces. He reported better result in egg detection with the use of polypropylene tubes compared with glass tubes in the procedure¹³. Present study also revealed that polypropylene tubes were significantly better compared with glass tubes, silicon-treated glass tubes and polyethylene terephthalate tubes. These findings indicated that some fecal mass containing parasite eggs adhere to the inner wall of the glass tube during FES procedure and this phenomenon is one of the factor associated with low recovery of parasite eggs.

Ether is regarded as a critical reagent in FES procedure. Ethyl acetate (flash point, -4°C; ignition point, 77°C) was used as a substitute for ether², and its efficacy has been confirmed¹⁹. Present study, however, showed that substitution of ether with ethyl acetate increased the amount of sediment that could cause low recovery of parasite eggs as mentioned earlier. Lawrence and Thomas²⁰ have also indicated that ethyl acetate would not make a good substitute because it could not dissolve the fat components in fresh feces. This would explain the increased amount sediment noted in the present study. Furthermore, fecal sediment obtained using ethyl acetate has been reported to contain debris and/or small liquid bubbles in the sample, which would obstruct observation of small sized parasites such as protozoan cysts²¹. On the contrary, Young *et al.*², reported higher recovery rate of *Giardia* cysts and *V. nana* eggs with the use of ethyl acetate than ether. Truant *et al.*¹⁹ and Erdman²¹, however, observed no significant difference between the two organic solutions in the recovery of parasites. Our result concurred with the result of Truant *et al.*¹⁹ and Erdman²¹. Although there is a report that suggests acetone is a suitable substitute for ether³, notable efficacy was not observed in our study.

Several researchers have modified FES procedure in the past^{2,3}. However, none of the modifications have been done by examining the entire steps of the FES procedure.

Therefore, this study re-evaluated the entire steps considering various factors that could influence the detection of parasite by this method. According to the results we obtained, FES procedure with the use of 10% PBFS at pH 7.0, three layers of gauze and polypropylene tubes improves the parasite egg recovery/detection in the fecal samples compared with the original FES procedure (Fig. 4). This modified procedure could detected 1.4 time more parasite eggs in the same amount of fecal samples than that of original FES procedure.

Based these findings, we compared the recovery efficiency of parasite eggs and the number of eggs recovered by both methods (the original FES procedure and the procedure we modified) using fecal samples collected from school children of intestinal parasite endemic areas in Nepal. However, no significant difference was found between these two methods in the recovery parasites in 112 fecal specimens examined. No significant difference in the results obtained by these two methods is thought to be caused by high intensity of parasite infection that occurs in endemic areas. However, the modified FES procedure was found to be superior in detecting the eggs of *V. nana*, hookworm, *T. trichiura* and *A. lumbricoides* to the original FES procedure. This result, therefore, suggested that the modified FES procedure appears to be effective in parasites in fecal samples in areas with low-intensity parasitic infection.

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FIGURES AND TABLES

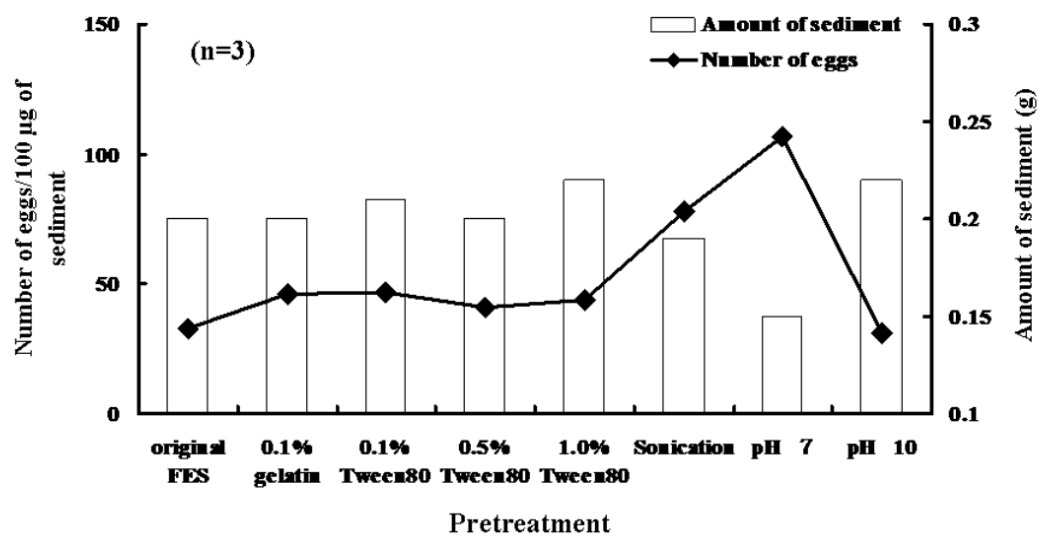


Fig 1. Effect of pretreatment of fecal suspension on recovery of sediment and eggs

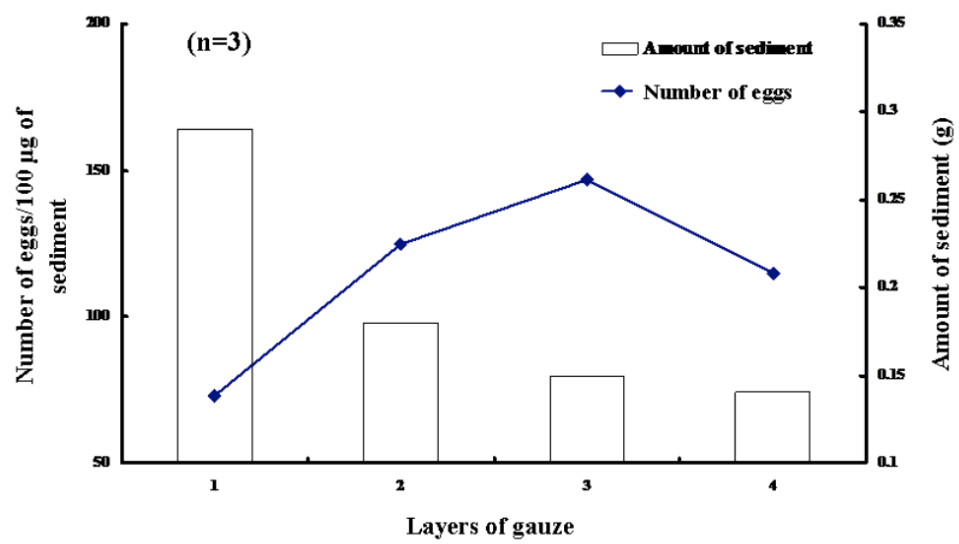


Fig 2. Effect of filtration by gauze on recovery of sediment and eggs

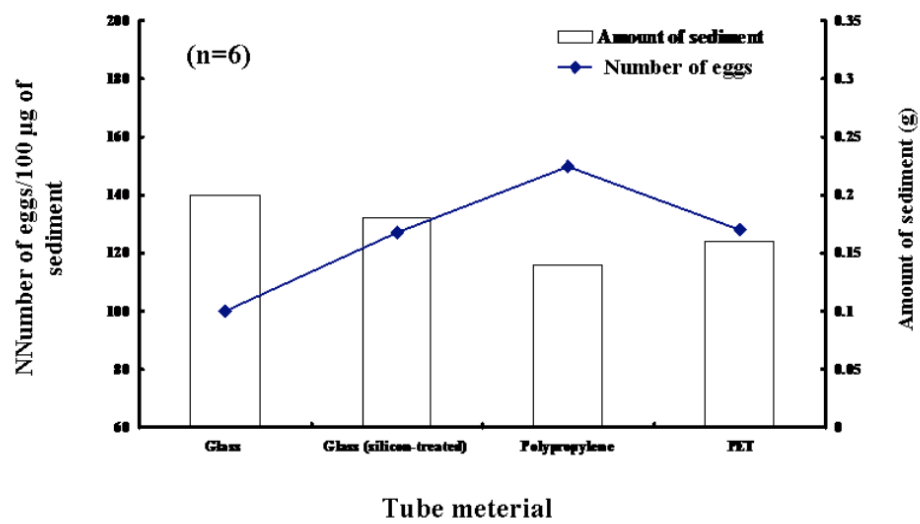


Fig 3. Relationship between test-tube material and efficiency of recovery of eggs

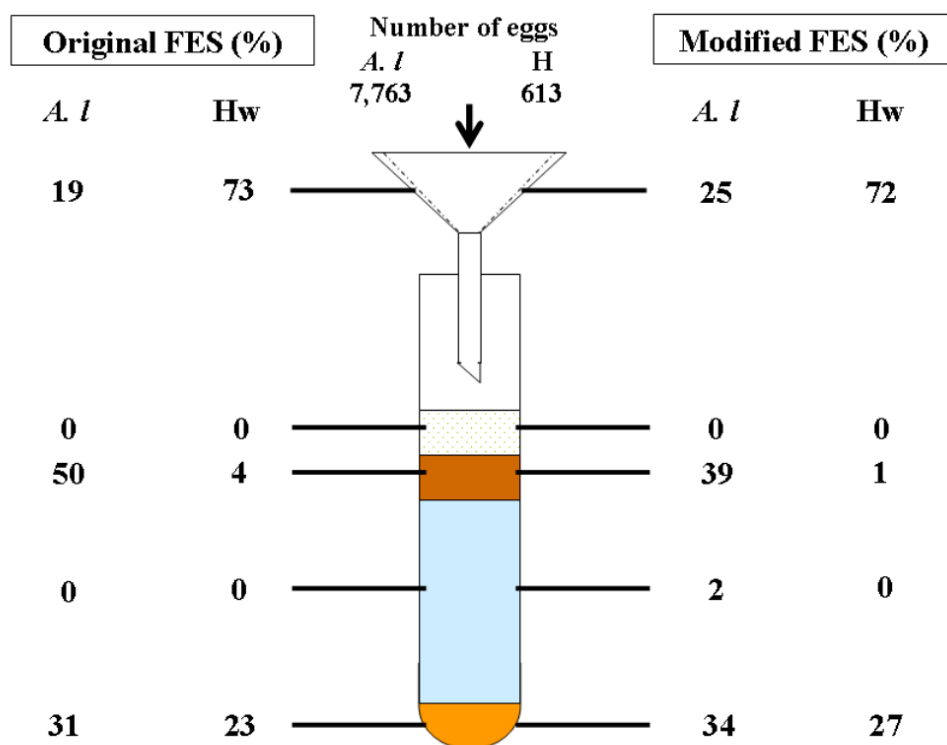


Fig 4. Comparison of egg loss during original and modified versions of the FES

Table 1. Usefulness of various types of organic solvents for recovery of *Diphyllbothrium latum* eggs in the formalin–ether sedimentation

Solvent	Weight of sediment (g)	Number of eggs*
Ether	0.14	136±17^a
Ethyl acetate	0.20	101±15^b
Acetone	0.26	94±23^c
Alcohol–ether mixture**	0.26	86±28^d
Xylene	0.20	123±25^e
Toluene	0.21	91±26^f
Methyl ethyl ketone	0.23	86±35^g

***Egg count per 100 µg of sediment. (n=4)**

****Same volume of alcohol and ether were mixed.**

Table 2. Comparison between the original FES and modified FES with regard to detection of the number of eggs in respective parasites

Species	Number of samples	Mean number of eggs collected	
		Original FES	Modified FES
<i>Vampirotepis nana</i>	8	197 (0–812)	300 (4–755)
Hookworm	6	22 (0–86)	29 (1–122)
<i>Trichuris trichiura</i>	22	16 (0–139)	23 (0–143)
<i>Ascaris lumbricoides</i>	1	142	222