

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

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ABSTRACT

The formalin-ether sedimentation (FES) method is considered as reliable method of fecal examination for the detection of parasites. In this study, we re-evaluated several aspects of FES such as (i) pretreatment of feces; (ii) filtration of fecal suspensions; (iii) test-tube material and (iv) substitution of ether by other organic solvents as to see an improvement in parasite egg recovery. The egg count was represented by the number of eggs detected per 100 µg of sediment. Pre-treatment of feces with formalin (pH 7) increased egg detection rate remarkably compared with original FES method. Use of three layers of gauze dramatically reduced the sediment in the final product, and led to an increase in the number of eggs detected. Use of polypropylene test tubes instead of glass test tubes also increased the number of egg detection. None of the organic solvents used to replace the ether produced better results. Based on these findings, we proposed a modified FES procedure. Further, we also compared the parasite positive rate and the number of eggs recovered by using original FES and the modified FES procedures by examining 112 fecal samples collected from school children of parasite endemic area in Nepal. Feces collected from Nepal had many parasite eggs, and these fecal samples barely displayed false-negative results even by method with low sensitivity. When the mean number of *Vampirolepis nana*, hookworm, *T. trichiura*, and *A. lumbricoides* eggs recovered by original FES and the modified FES methods was compared, the values obtained by modified FES were superior (higher). This result suggested that the modified FES is effective and better for the recovery of parasite eggs in areas of low-intensity parasitic infection.

Keywords: Fecal examination, formalin-ether sedimentation, parasite eggs, recovery efficiency.

INTRODUCTION

Various methods can be used for the detection of parasites in fecal samples: direct smear, flotation and centrifugal sedimentation. For the detection of helminth parasite 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 direct smear method and other centrifugal sedimentation methods in the recovery of parasites (helminth eggs and protozoan cysts). Since then, FES method has undergone various modifications,²⁻⁴ 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 have been noted in original FES method. For example, Ritchie¹ did not mention precise name of parasite despite detailed descriptions of FES procedures. Similar problem was 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, also came 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 results were not considered.^{2,3} Reports discussing FES method in a comprehensive manner are 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 might 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 This has 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 (65 years ago) has not been re-assessed for the detection of parasites in fecal samples in present 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 Nepal where intestinal parasitosis is endemic.¹³

MATERIALS AND METHODS

Examination of the basic conditions in the respective processes of FES

Preparation of fecal samples: For accurate measurement of the efficiency of egg recovery, specified (known) number of *Diphyllobothrium 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 recorded. The volume of suspension examined was such which produced 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 (collected from zoo) 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.

Re-examination of FES procedure: The original FES procedures of Ritchie¹ and Price¹⁴ 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 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. We re-evaluated all four steps of original FES method i.e. (1) pre-treatment of feces, (2) filtration, (3) test-tube material and (4) substitutes for ether, and examined the optimal conditions for these components. The original FES procedure was carried out, with modifications made to each of these four steps using a standard fecal 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 included, addition of (i) 0.001% gelatin in 10% formalin solution, (ii) 0.001%, 0.05%, and 0.01% Tween 80 in 10% formalin solution, (iii) adjustment of pH of 10% formalin solution to 7 (pH 7) or 10 (pH 10) and (iv) Three times sonication (20-s) of 10% formalin/feces suspension.

(b) *Filtration:* One, two, three and 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; Asona, 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 recovery rate by FES procedures

Once the original FES and modified FES methods

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

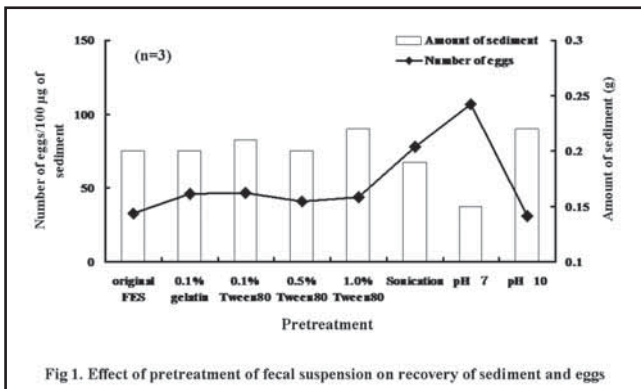
Comparison of egg detection from fecal samples

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

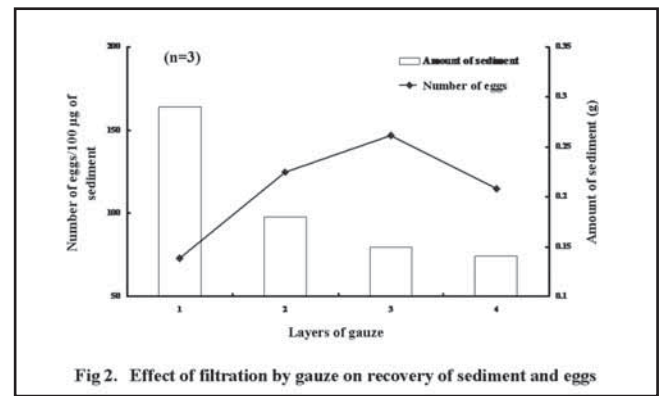
Examination of the basic conditions in the respective processes of FES

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

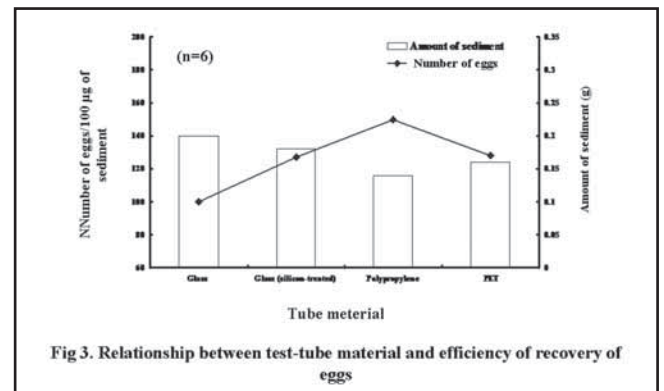


Use of formalin solution with added gelatin or different concentrations of Tween 80 did not improve the recovery of both fecal sediment and eggs. Pre-treatments such as sonication or adjustment of pH at 10 also did not improve the recovery either. In contrast to these findings, adjustment pH of the fecal suspension at 7 (pH 7) dramatically reduced the amount of fecal sediment, which led to a significant increase in the parasite egg recovery (count) compared with the original procedure (i.e., 33 eggs/100 µg of sediment by original FES method whereas 107/100 µg of sediment; p<0.05).

(b) *Filtration*: Fig. 2 shows the effect of filtration on the recovery of fecal sediment and parasite eggs. In this examination, filtration was done using one, two, three and 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 reduction in fecal sediment. Therefore, we concluded that filtration of samples using three layers of gauze was optimal.



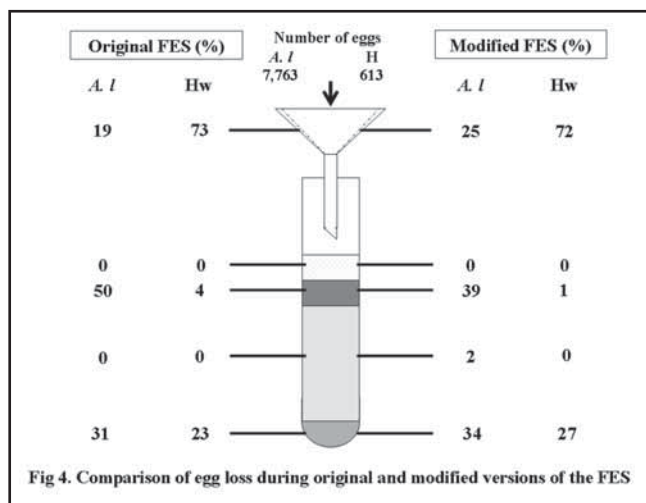
(c) *Test-tube material (type)*: Of the different types of tubes used in the procedures the recovery of eggs in the sediment was significantly higher when polypropylene tubes were used instead of glass tubes (p<0.05) as well as other tubes (Fig. 3).



(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). However, none of these proved to be the better than ether in obtaining a minimum amount of sediment (the final product) and parasite egg recovery. Instead, ether was better than all these substitutes but the difference was not significant (p>0.05).

Comparison of egg recovery rate by FES procedures

In this experiment, human feces with 15,525 fertilized eggs of *A. lumbricoides* and 1,225 hookworm eggs was used. When this was subjected to original FES procedure, few *A. lumbricoides* and hookworm eggs were found in ether and formalin layers while most of the eggs were found trapped in gauze, fecal layers and sediment. Of the total, only 31% of *A. lumbricoides* eggs and 23% of hookworm eggs were found in the sediment. In modified FES procedure with three layers of gauze also eggs were found to be trapped in the gauze and in fecal layer. But, when compared with the original FES method, the modified FES method yielded less amounts of sediment (less by 14%) and this made easy in microscopic observation/parasite egg detection. The number of eggs in the sediment, therefore, 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, *V. nana*, hookworm, *T. 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 FES but positive by modified FES had egg of *T. trichiura*, 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 changes compared with other methods such as floatation and/or Kato-Katz methods. To evaluate the FES method, we considered four steps/factors in the procedure focusing on the amount of sediment produced and the number of egg recovered.

Approximately 200 µl of sediment was obtained by original FES procedure out of which 20 µl 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 Price,¹⁴ 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 (pH 7), the efficiency of egg recovery was increased compared to original FES methods. This result was consistent with that of Richie *et al*,¹⁵ who demonstrated the effect of pH in egg recovery. Richie *et al*¹⁵ and Oshima *et al*,¹⁶ however, stated that the optimal pH level varied according to parasite species. As the study was targeted only to hookworm eggs so we cannot draw conclusions about the effect of pH on recovery of other helminths parasite eggs.

In general, gauze is used for filtration of fecal suspensions in FES procedure to remove bigger particles. Previously, filtration of fecal suspensions with one layer of gauze was recommended by Takahashi *et al*¹⁷ and Nakanishi *et al*¹⁸ whereas by Ritchie¹ and Young *et al*² recommended two layers. However, in this study, use of three layers of gauze yielded highest number of parasite eggs. Gauze is expected to trap most of the fecal particles larger than parasite eggs and therefore, make easy for microscopic observation. Pamba and Mulega¹⁹ 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, therefore, 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).

Price¹⁴ 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 procedure.¹⁴ 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 and ignition point: 77°C) was used as a substitute for ether,² and its efficacy has been confirmed.²⁰ 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 Thomas²¹ have also indicated that ethyl acetate is not 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 cysts.²² 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*²⁰ and Erdman,²² 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*²⁰ and Erdman²². Although there is a report that suggests acetone is a suitable substitute for ether³, however, 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 all four steps of the FES procedure. Therefore, this study re-evaluated the entire

steps considering various factors that could influence the detection of parasite in fecal specimen by the FES method. According to the results we obtained, FES procedure with the use of 10% formalin solution with pH 7.0 (phosphate-buffered-formalin-solution), 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 detected 1.4 times more parasite eggs in the same amount of fecal samples than that of original FES procedure.

Based on 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 112 fecal samples collected from school children living in intestinal parasite endemic area (Nepal).¹³ However, no significant difference was found between these two methods in parasite eggs recovery in these fecal specimens. This might have attributed to 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 detecting the parasites in fecal samples collected in areas with low-intensity parasitic infection.

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