An advanced protocol for the purification of whipworm eggs from feces for use as therapeutic agents

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ABSTRACT

Recent studies have attempted to treat autoimmune diseases using *Trichuris suis*. whipworm) eggs. Large quantities of eggs can be obtained efficiently by collecting from the feces of the porcine hosts rather than by extracting from the female worm uterus. However, it is difficult to process large amounts of feces using the current methods. In the present study, we propose a method to collect the eggs from bulk feces more efficiently. Collecting the eggs using washing meshes (25 μm sieve) yields 65.7% (56.0–70.7) of eggs (median, min-max) from 100 g feces. Our method, which uses ethyl acetate and simulated gastric fluid, yielded 91.4% (91.4–94.0) of the eggs from 100 g feces into the separated aqueous solution. Egg collection using simulated gastric fluid (SGF) method was also 60 min faster than that using the sieve method. As the SGF used in the experiment is a strongly acidic reagent with a pH of 1–2, embryonation of the eggs was induced by the rapid pH change. As a result, 37.1% (8.0–77.8) of the eggs had embryonated two months after SGF stimulation. Using the developed method, we could process the feces quickly and efficiently. Furthermore, after purification, egg embryonation could be induced without any harmful reagent treatment. This method is expected to be helpful for further research using *Trichuris suis* eggs.

1. Introduction

There is currently no definitive treatment for inflammatory bowel disease (IBD), a type of autoimmune disorder [1,2]. Treatment of IBD using *Trichuris suis* (whipworm) embryonated eggs has been attempted. [3,4]. In order to investigate the anti-IBD effects of the eggs and to use them therapeutically, it is necessary to establish a mass separation and rapid collection method for the eggs produced by *T. suis* adults. However, when female adults are isolated from their porcine hosts, it is difficult to obtain a sufficient amount of eggs. Also, most of the eggs collected from the parasite uterus at that time contain undeveloped embryos [5]. Methods for separating nematode eggs from soil or stool using sieves have already established, with recent attempts to improve egg quality and quantity [6]. Tsubokawa et al. collected eggs from *P. westermani*, *C. sinensis*, and *M. yokogawai* using a stainless-steel mesh from 100 g of stool [7]. However, the stainless-steel mesh has a limited capacity to deal with the fecal matter as stool debris easily clogs the holes in the mesh. It has to be rinsed with a significant amount of water

and the eggs collected from the debris. Flushing out the water from the stainless-steel mesh also takes a considerable amount of time and effort. While a new approach using improvement on the flotation method for obtaining parasite eggs from soil has also been developed [8], that method is not suitable for processing large amounts of feces. Therefore, it is necessary to establish a method for quickly obtaining eggs in large quantities from stool rather than from the adult worm's uterus of the adult worm.

In the present study, we established a method to collect *T. suis* eggs efficiently using weak acidic ethyl acetate, modified simulated gastric fluid (SGF), and a PluriStrainer set. This improved isolation method will facilitate the investigation and treatment potential of whipworm eggs for autoimmune diseases.

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2. Material and methods

2.1. Collection of T. suis eggs from infected pigs

Eleven Yorkshire pigs were obtained from local pig farms (Yangsan, Korea). The weight and age of the pigs on arrival were 15.3 kg (14.4–16.2) and were 40.6 days (30.4–50.8) old, respectively (median, min-max). Helminth infection of the Yorkshire pigs was detected by the Medical General Laboratory (MGL) method [9]. The maintenance of pigs followed the Institutional Animal Care and Use Committee (IACUC) of the Pusan National University Yangsan Hospital (IACUC protocol approval number: PNUYH-2017-047). Of the 11 Yorkshire pigs, six pigs were used main-experiment (3 pigs on each experiment, repeated) and five pigs were used on pre-experiment, The pigs were weighing 22.0 kg (19.9–24.1) and of 61.2 days (51.6–70.8) old (median, min-max), as no other parasitic eggs were observed in their fecal samples. Three pigs were orally administered 10,000 T. suis eggs once with food, milk bread used for the food used and 10,000 T. suis eggs suspended in PBS poured into the milk bread [10]. Cut the bread to the right size to eat until the pig wholly consumed and eggs were observed in the stool at least 35–59 days later. Another three pigs were given 10,000 T. suis eggs each to induce an infection. Feces collected to isolate eggs were stored at 4 °C until the test. All eggs could be separated from the feces within 2 h. Pig stools were examined under an optical microscope (Zeiss Axioskop2, Thornwood, NY, USA) to confirm the presence of T. suis eggs.

We calculated the number of eggs per gram (EPG) in the fecal material. The modified Ritchie concentration method was used for the determination of EPG from sediment [11]. Briefly, one gram of solid stool or five gram of fluid stool grams of feces were sampled using wooden applicator sticks (HAN YANG, Korea, 150 mm × 2.2 mm, NO. SW200PO) from the feces previously refrigerated. The stool was liquified by adding 10 ml of physiological saline. It was then filtered by two layers of moist gauze and collected in a test tube. After centrifugation for 1 min at 900 ×g, the supernatant was discarded and the solid resuspended in saline. Ten ml of 10% formalin was added to the precipitate and allowed to stand for 5 min. Then 3 ml of ethyl acetate was added and the solution shaken until all of the sediment was floating. After centrifugation at 900 ×g for 1 min and 30 s at 25 °C, the layer formed between formalin and ethyl acetate was separated from the wall of the test tube and discarded with the supernatant, and the precipitate inspected. Microscope slides (25.4 mm × 76.2 mm, cat.no.7101, Henso, China) were filled with 10 μL of the mixture using a pipette, gently covered with a cover glass (22 mm × 22 mm) and the number of eggs in the cover glass counted. The number of parasitic eggs per gram was combined between two cover glass estimates, as the results of the two cover glasses scaled 0.02 ml to a total of 1 ml, approximately a rate of 1 / 50th. As such, the number of eggs was multiplied by 50 to give a value per ml. However, since the experiment often started with 5 g of liquid stool, the number of eggs counted was divided by 5 to provide an egg calculation per 1 g of stool. The formed stool was collected only 1 g and so was the correct amount for measurement. The EPG was assessed in triplicate and the number of eggs separated from the feces was immediately counted and recorded. The average of the calculated EPGs was determined as 117 (72–191), 2864 (2695-4029) and 4795 (2776-5636).

From the EPG test it was found that the highest number of eggs were collected from the feces after 91 to 97 days of infection when present in the feces. As such, the egg collection was repeated three times with 100 g of feces each time after 91–97 days, and the collected feces purified to obtain the most eggs. The feces were divided into two samples of 100 g each. One sample was used to separate the eggs using the stainless-steel sieve method. The other sample was subjected to the SGF method plus sucrose separation.

2.2. Egg separation from feces using the sieve method and storage for embryonation

Each sample of 100 g of feces from the three pigs was suspended in five volumes of distilled water and then filtered through two layers of gauze. Each of the 500 ml samples of fecal suspension was then filtered through a series of stainless steel sieves (Chung gye sang gong sa, Seoul, Korea, Ø 200 mm × 45 mm) with pores ranging from 200 to 25 μm in diameter (200, 106, 75, 53, 38, and 25 μm). The suspension was washed with 1 L of distilled water per stainless steel sieve it passed. When filtering feces using meshes with different pore sizes, the debris cannot escape from the mesh and blocks the pores in each layer. Therefore, it is necessary to tap both sides of the mesh to remove the suspended matter from the pores, especially during the 1 L wash step. Sieve-washed water was collected and centrifuged at 3220 ×g for 5 min at 25 °C to collect the eggs. Finally, the eggs captured on the 25 μm mesh were collected and centrifuged at 415 ×g for 5 min at 25 °C for egg embryonation rates. As in the EPG method, the number of eggs obtained for each sieve counted three times. The eggs were resuspended in 1–5 ml of phosphate buffered saline (PBS) and stored in a 15 ml conical tube at 25 °C. The eggs stored in PBS were divided into three batches and stored.

2.3. Egg separation from feces using the SGF method and storage for embryonation

The 100 g of pig feces were suspended in 250 ml of distilled water and filtered through gauze (two layers). The suspended fecal matter was placed in specialized bottles in a centrifuge (3141–0500, 500 ml, Nalgene™ PPCO Centrifuge Bottles with Sealing Closure, Seoul, Korea) and 75 ml of ethyl acetate was added to the extract. The bottle was shaken well to mix the two solutions and then centrifuged for 5 min at 3220 ×g at 25 °C. After centrifugation, the separated solution in the upper layer was discarded. Then, 25–40 ml of distilled water was added to resuspend the remaining pellet in the bottom of the bottle. The resuspended pellet was transferred to a 50 ml conical tube (#50050, SPL, Gyeonggi-do, Korea) and centrifuged for 5 min at 3220 ×g at 25 °C. All but 5 ml of the supernatant was discarded, and the pellet was resuspended in this 5 ml of distilled water. Next, 45 ml of SGF [1% pepsin from porcine gastric mucosa (P7000-100G, Sigma, St. Louis, United States) and 1% hydrochloric acid (H0519, SAMCHUN, Seoul, Korea) in distilled water] was mixed with 5 ml of the pellet (9:1 ratio) and allowed to react for 15 min at 37 °C. The solution was poured into stainless steel sieves with mesh sizes of 53 and 25 μm and washed with 1 L of distilled water. After that, the 53 μm sieve was removed, and the 25 μm sieve was washed with 50–500 ml of distilled water sprayed from the opposite side of the washed surface of the sieve, and the eggs were collected in a 50 ml conical tube.

The PluriStrainer set was composed of a connector ring (41–50,000-03, PluriStrainer, Germany), a 20 μm woven mesh (43–50,020-03, PluriStrainer, green, Germany), and a funnel (42–50,000-03, 24 ml, PluriStrainer, Germany). The PluriStrainer set was placed over a fresh 50 ml conical tube. The eggs were purified using PluriStrainer set according to the manual. After purification of the eggs, 20 ml of a 100% solution of sucrose (Saccharose, Junsei, Japan) was added into the solution. The egg suspension was centrifuged for 5 min at 3220 ×g at 25 °C. The sucrose boundary layer containing the floating egg solution was collected using a 1 ml pipette. Subsequently, The eggs were resuspended in 1–5 ml of phosphate buffered saline (PBS) and stored in a 15 ml conical tube at 25 °C. The eggs stored in PBS were divided into three batches and stored.

The SGF method involves floating the feces in ethyl acetate, in which the eggs sink to the bottom of the tube and can be separated from large particle debris, including oil components. Thus, This step resulted in almost no fecal residue appearing on the two gauze layers.

2.4. Observation, management of the egg conditions and statistical analysis

The stored eggs were inspected three times using an optical microscope on the first, second and third months after extraction. An optical microscope was used to count the number of eggs counted monthly using one of three 15 ml tube batches and to observe any morphological changes between the eggs purified using the sieve and SGF methods. The eggs were classified as embryonated eggs when larvae were present in the eggs, determined by the morphological changes in the eggs, while the forms in which blastomeres were observed were classified as non-embryonated eggs. Finally, if the larvae were in a collapsed form or if bubbles had formed, they were classified as degenerated eggs. Each tube was counted three times. The eggs in the 15 ml tube were shaken gently to resuspend them in the solution. The specimens were examined at x40, x100, and x200 magnification under the optical microscope. There was no specific device used to inject oxygen. The determination of morphological state change of the eggs was as described by Beer [12].

To determine the statistical significance, the separated embryonated eggs were counted following extraction by the sieve or SGF method. The number of each type of egg was calculated by accumulating the number of embryonated eggs counted every month before classification. Statistical analysis was performed using GraphPad Prism software (version 7) using an ANOVA test where $P < .05$ was considered statistically significant. The graphs were prepared in Excel 2016 and GraphPad Prism.

3. Results

For the sieve method, which included washing with distilled water at every sieve stage, it took approximately 120–240 min to process the feces, which yielded 11,700 (7200-19,100), 286,400 (269,500-402,900) and 479,500 (277,600-563,600) eggs from 100 g (median, min-max). This process was performed three times for feces from each pig. Yielded, 7792 (4033-11,557), 188,285 (157,175-284,875) and 315,233 (162,500-390,522) eggs were collected at 25 um sieve. The percentage of total eggs counted on each sieve was 2.4% (1.8–3.4, at 200 μm), 2.8% (2.5–3.6, at 106 μm), 3.1% (2.8–3.4, at 75 μm), 0.1%

(0.02–0.1, at 53 μm), 1.1% (1.06–1.2, at 38 μm), and 65.7% (56.0–70.7, at 25 μm), of which 11.3% (6.5–16.5) of eggs were collected in the filtered fluid. For the SGF method, it took approximately 60 min to treat the feces containing 11,700 (7200-19,100), 286,400 (269,500-402,900) and 479,500 (277,600-563,600) eggs in 100 g (median, min-max), resulting in 10,694 (6581-17,958), 262,780 (246,333-378,266) and 438,281 (253,737-525,152) eggs being collected, and was also performed three times from each pig. In the 53 μm and 25 μm sieves used in this method, 0.08% (0.08–0.10) and 96.8% (89.4–99.3) of the eggs were present on each sieve, respectively (Fig. 1). Finally, 91.4% (91.4–94.0) of eggs were recovered from the sucrose layer (Supplementary Tables 1, 2).

Using the sieve method, up to 65.7% (56.0–70.7) of the eggs could be collected with the 25 μm sieves, compared with 96.8% (89.4–99.3) using the SGF method. In the sieve method, nearly all of the fecal waste was present at the gauze stage. Considering that the eggs are typically 50 μm in width and about 20 μm in height, we believed that some of the eggs would be retained by the 53-μm-thick stainless-steel sieve if they were in the horizontal plane, but those in the vertical plane would pass through. Furthermore, during washing, the eggs could change their orientation and pass through the 53 μm sieve. However, using a 25 μm stainless steel sieve, most the eggs would be caught by the mesh and recovered.

In the SGF method, the solution passed through a 53 μm sieve, a 25 μm sieve, and a 20 μm woven mesh in the PluriStrainer and was then viewed under an optical microscope. When observed under the microscope, no particles smaller than the eggs were seen in the woven mesh, and only particles much larger than the eggs were present (Fig. 2 C). The 20 μm PluriStrainer has a particle size limit similar to the size of the eggs. When the eggs were subjected to a sucrose solution treatment and centrifugation, particles larger than the eggs were pelleted and only the eggs remained in the boundary layer. Thus, the eggs collected from the boundary layer were pure (Fig. 2 D). Therefore, 165 g was determined as the most suitable amount of starting fecal material as it resulted in only a light covering of the 20 μm woven mesh.

In the samples treated using only sieves to separate the eggs from the feces, embryonated forms were not observed after two months of storage at 25 °C. After three months, however, 2.8% (1.4–4.8) of the

Fig. 1. Comparison of egg isolation from pig feces using the sieve or simulated gastric fluid (SGF) methods. Using the sieve method to isolate eggs from feces, 65.7% (56.0–70.7) of the eggs were recovered in 120–240 min at 25-μm stainless steel sieve. By contrast, when the eggs were separated using the SGF method, the recovery rate was 91.4% (91.4–94.0) in sucrose, and the time required was about 60 min. The time taken to isolate eggs from the stool varies depending on the stool status (e.g., formed, soft, liquid). After processing the feces, the recovered egg suspension at each step was observed using an optical microscope (See Figs. 1, 2 A–D). Using the feces of 3 pigs, the eggs obtained by passing through the sieve of each step were counted three times and represented as a percentage of total eggs observed in the feces. Data are median (min-max).
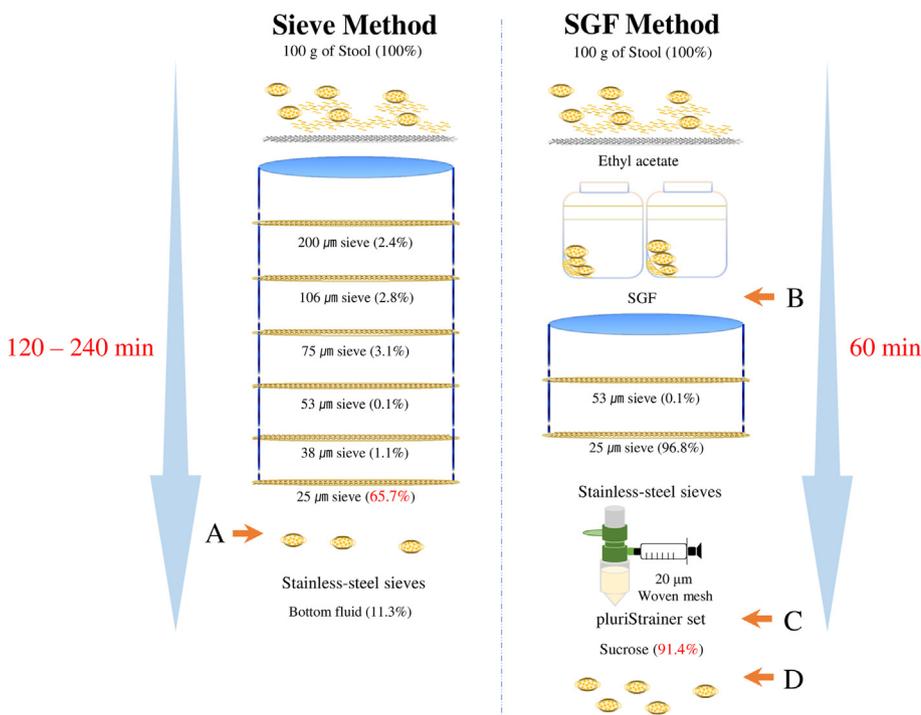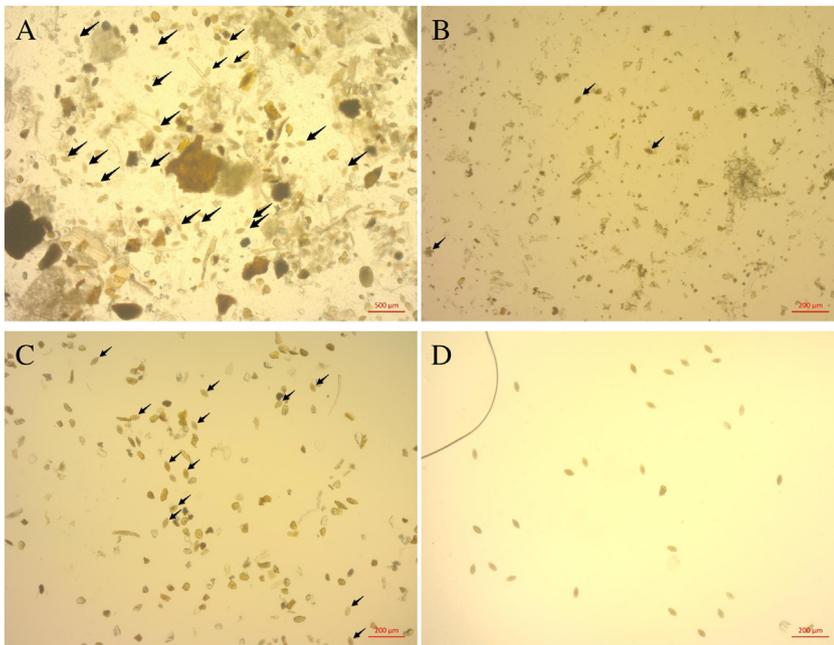
Fig. 2. Mesh residue after fecal treatment observed under an optical microscope at each step at each step of the sieve and SGF recovery processes. (A) After fecal processing was completed, large lumps of particles were seen mixed with the eggs (x25). (B) Floated eggs after applying the ethyl acetate and simulated gastric fluid (SGF) and a 25 μm stainless steel sieve (SGF method) (x50). (C) Floated eggs obtained used the SGF method with filtering through a 20 μm PluriStrainer (x50). (D) Eggs separated by sucrose using SGF method. Black arrows indicate *T. suis* eggs (x50).
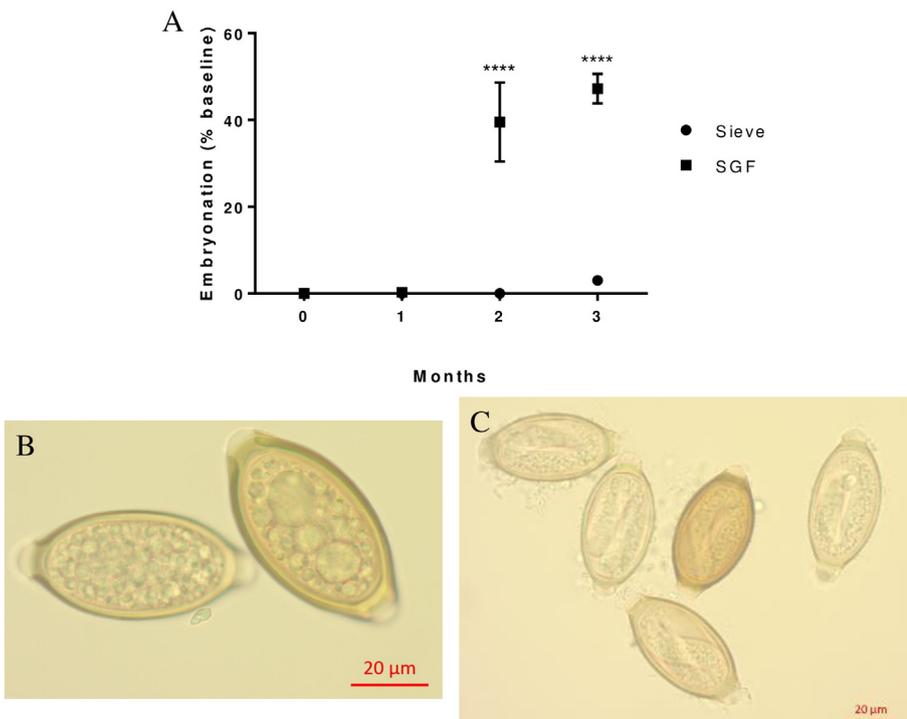
Fig. 3. Comparison of embryonation of eggs isolated by the sieve and simulated gastric fluid (SGF) methods. (A) When the eggs were separated by the sieve method, larval forms were not observed inside the eggs after two months. At the third month, however, 2.8% (1.4–4.8) of the eggs were in embryonic development (median, min-max). Using the SGF method, 37.1% (8.0–77.8) of the eggs were embryonated at two months and 47.8% (29.0–65.0) at three months. (B) The appearance of eggs immediately after isolation from the feces by the SGF method (x400). (C) Eggs incubated for two months at 25 °C after separation using the SGF method (x400). For panel (A) $n = 9$ and the errors bars indicate the mean ± SEM. ****$P < .0001$, assessed by two-way ANOVA. Post-test was performed with Bonferroni's multiple comparisons test. *$P < .05$ was considered statistically significant.

eggs had embryonated. Using the SGF method, 37.1% (8.0–77.8) of the eggs at the second month and 47.8% (29.0–65.0) of the eggs at the third month had embryonated (Fig. 3 A). Immediately after separating the eggs from the feces, no embryonated eggs were observed (Fig. 3 B). After two months, some larvae had formed in the eggs after the blastomere phase (Fig. 3 C).

4. Discussion

In this study, we have developed a rapid method for separating *T. suis* eggs from swine feces. In the past, eggs have mainly been isolated from feces during autopsies and significant amounts of time, effort, and separation reagents were required to treat the feces. Another method

using a single or multi sieve to separate the eggs was established [5,13–20], such as for isolating *Toxocara* species eggs from soil or feces [21]. Another study reported a unique way to recover parasite eggs from the soil [8]. However, the previously reported studies did not quantify the number of eggs collected. In the present study, we confirmed that different numbers of eggs were recovered according to the diameter of the sieves used in the experiment, and, thus we developed a more efficient method to purify the eggs.

It was difficult to detect the presence of the eggs under the optical microscope when collected using the sieve method as the final product contained a large amount of fecal and particle residue (Fig. 2 A). The sieve method showed an egg recovery rate as low as 65.7% (56.0–70.7), but even when the eggs were treated with 100% sucrose, the recovery

rate was almost unchanged. Many eggs may be lost with each sieving; therefore, the fewer rounds of sieving, the better. Consequently, in the SGF method, we selected only the 53 and 25 μm meshes, which can collect the maximum amount of eggs while minimizing the time taken for sieving. The more significant the amount of residue and the more substantial its size, the lower the rate of recovery of the eggs, with the recovery found to drop to as low as 50% (data not shown). When solid stool was loosened in Distilled Water (D.W), the stool suspension becomes awash with fine particles in the sieve of 53 μm, less than the liquid stool. Once the sieve is blocked, the suspension cannot pass through. At 53 μm, 38 μm and 25 μm, the suspension does not pass the sieve, especially at 38 μm, and so the water does not entirely flow through the sieve, even if it is left for three days. Therefore, sieves smaller than 53 μm must be worked by hand until the suspension exits the sieve entirely. This procedure applies to all sieves used in the experiment, but especially smaller than 53 μm mesh, and so the sieve should be strong and long lasting. Therefore, it is correct that the number of eggs collected increases as mesh size decreases. However, it takes more than twice as long to completely remove the suspended solids for 53 μm, 38 μm, and 25 μm mesh, when compared with the time required for the suspension to flow through a sieve of 53 μm or larger. The few eggs were collected from the 38 μm sieve > 200 μm sieve. This process must be repeated for each mesh size, and significantly slows the separation.

After SGF treatment, we confirmed that a significant number of large particles were removed from the suspension. However, some smaller particles were still present. The sizes of the residual particles were smaller than those in the eggs processed by the sieve method (Fig. 2 B). Using the SGF/sucrose process, we could obtain more purified eggs than the sieve method. In the 20 μm woven mesh, the accumulation of more substantial amounts of residue over the top of the layer can form a residue mass. This residue mass was identified in the layer formed between the sucrose and distilled water. Both methods used in the experiment did not achieve 100% recovery when the number of eggs present in the feces and the number of eggs gathered at the bottom through the sieve were compared during each stage of processing. This fact reflects that eggs remained on the sieve during the processing of the feces.

The SGF method resulted in embryonation of the separated eggs. A previous report suggested that changing the egg storage temperature can accelerate embryonation [22]. In that report, the eggs were stored at pH 1 using a sulfuric acid solution. In our experiment, SGF similar to gastric fluid was used, which has a pH of 2. The SGF method eliminated the coarse particles, leaving only those smaller (Fig. 2 B). In addition, the SGF method resulted in 47.8% (29.0–65.0) embryonated eggs after three months, with no degeneration. Embryonation can be delayed without an individual oxygen supply. However, when inspecting the eggs, it is believed that air was introduced to some extent due to the opening in the 15 ml tube lid. Furthermore, oxygen was introduced when the PBS was pipetted to mix the eggs for the test. Oxygen is one of the most important factors influencing the development of nematode eggs in many host species [23,24]. The viability of *Ascaris suum* and *Oesophagostomum dentatum* eggs has been reported to be affected by changes in pH or temperature of the fecal environment [23]. Therefore, pH is thought to affect the survival of eggs. Here, we carefully deduced that pH also affects egg embryonation and hope to verify this by experiment later. Using the SGF method, we can greatly shorten the time taken to treat feces, when compared with the sieve method, and increase the efficiency of egg collection. Furthermore, the eggs obtained from the final product could be embryonated without any further treatment. Therefore, using the SGF method, it is only necessary to store the eggs at a temperature suitable for embryonation. Our developed method will be useful for future studies using *T. suis* eggs.

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Conflict of interest

The authors declare no conflict of interest.

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