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USE OF SEMI-HYDROLYZED COLLAGEN FOR EXTRACTION OF L1 OF *ANGIOSTRONGYLUS CANTONENSIS* FROM RODENTS FECES

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Abstract: Larval purification techniques encompass methods that depend on the biology of the organism, its matrices and objectives. In this context, fecal samples are difficult substrates to handle due to the presence of particles that can compromise their use for research into various pathologies. This study presents a technique using semi-hydrolyzed collagen for the purification of larvae from feces of rodents experimentally infected with metastrongyles.
Keywords: *Angiostrongylus*; purification of larvae; semi-hydrolyzed collagen; feces.

INTRODUCTION

Larvae purification processes are common in the field of Parasitology and cover a wide range of methods that depend on the biology of the organism, matrices where they are found and objectives to be achieved with their isolation. In food microbiology, for example, concentration techniques are carried out to evaluate the presence of larvae in vegetables and guarantee the quality of the food for human consumption. On the other hand, in clinical analyses, parasitological examinations are carried out with the aim of observing the presence of pathogenic microorganisms in biological samples such as blood, cerebrospinal fluid (CSF) or feces with the aim of confirming the diagnosis of parasitic diseases.

In this context, fecal samples represent a difficult substrate to handle due to the presence of food debris, microbiological organisms, mucus, among other particles and substances that may be present. When the objective is merely the diagnosis of parasitic diseases, existing methods, such as sedimentation by Hoffman, Pons and Janer (1934) or positive hydrothermotropism by Rugai et al. (1954) and Baermann (1917), are very sensitive for the observation of helminth larvae. However, if the intention is to obtain specimens of larvae for molecular biology research and examinations, the presence of a large amount of impurities can make the procedures to be adopted difficult and/or expensive, even compromising the results.

Some methods used to isolate larvae are based on helminth biology. Positive hydrothermotropism, for example, is the basis for the extraction of larvae by the method of Baermann, Rugai and Harada Mori (1955), however, a large amount of sediment is also recovered in these methods. Cultivation of larvae on granulated charcoal or culture medium, described by Koga et al. (1991), can be used to obtain geohelminths, taking advantage of the geotropism of this group of parasites. But, like previous techniques, the presence of impurities is inevitable. In these cases, low speed centrifuges contribute to the elimination of lighter particles and filtration with gauze can retain larger particles, providing physical barriers and reducing, but not preventing, the formation of unwanted sediments.

In the case of metastrongyle larvae, such as *Angiostrongylus cantonensis*, this type of tropism does not seem to exist, as they present wave-like movements that do not necessarily lead them in any specific direction. In fact, from a biological point of view, these evolutionary forms need to remain in the feces of their definitive hosts (rodents) so that their cycle continues in intermediate hosts (snails, snails and slugs).

This way, this work aims to present an alternative for the isolation of helminth larvae, from rodent feces, to eliminate or significantly reduce the presence of fecal debris and, thus, simplify and relieve experiments that require evolutionary forms with high degree of purity, such as extractions of proteins or DNA from the parasite.

MATERIAL AND METHODS

OBTAINING L1 FROM *ANGIOSTRONGYLUS CANTONENSIS*

Stool samples containing L1 larvae of *A. cantonensis* were obtained from the evolutionary cycle of the nematode maintained at the Enteroparasites Center of the Adolfo Luz Institute, which uses wistar rats as definitive hosts and *Biomphalaria glabrata* as an intermediate host (Mota et al., 2020).

To isolate the larvae, fecal samples from three rats with 61 days of infection and three with 132, collected overnight, were grouped and subjected to the Rugai method, with dechlorinated water, as described in Mota et al. (2020) and, from the pellet formed, purification was carried out with semi-hydrolyzed collagen.

PREPARATION OF SEMI-HYDROLYZED COLLAGEN

The choice of collagen from chicken feet was based on its low cost, easy processing and high yield and the procedures followed the methodology used by Almeida (2012). However, modifications were made that simplified the extraction steps. To this end, 500g of chicken feet had their nails removed, washed in running water and left immersed in 4% acetic acid for 3 hours to reduce the calcium content. They were then washed again in running water three times, cut into small parts and placed in a 1000mL beaker containing 340mL of distilled water. The material was cooked on a thermal plate, where the content temperature was maintained at 92ºC for 90 minutes. Then, the material was strained through filter paper folded into a cone, with a generous wad of cotton wool inside. Filtration was carried out in a 500mL beaker and the filtrate was transferred to a container with a lid, which was left at room temperature until complete cooling and solidification (Figure 1).

After the cooling process, the column of fat accumulated on the surface of the collagen gelatin was removed with the help of filter paper and the surface area was washed with distilled water three times, with excess water also removed with filter paper. The product was stored under refrigeration until use, not losing its properties for purifying larvae until it was completely exhausted (60 days). The volume of raw collagen obtained (approximately 300mL) presented a density between 1350 and 1475, measured at 20ºC. This variation did not interfere with the L1 purification process of *A. cantonensis*.

Figure 1: Collagen extraction. A: material used; B: controlled heating of chicken feet fragments; C and D: filtering; E and F: collagen gelatin. Source: Melo, 2019.

PURIFICATION OF L1 LARVAE FROM *A. CANTONENSIS* WITH SEMI-HYDROLYZED COLLAGEN

Larvae were obtained from rodent feces using the Rugai method, carried out with dechlorinated water at room temperature.

To analyze the efficiency of the purification process, the Rugai sediments were evaluated at two times, 2 and 4 hours, in the same experiment, represented in two stages. To this

end, 10mg of collagen gelatin was liquefied at 37ºC and diluted with 10mL of dechlorinated water. The product was placed in a Petri dish and refrigerated for 1 hour, until gelatin was formed.

In the first stage, the feces concentrate obtained after 2 hours of Rugai sedimentation, approximately 500µl of *pellet*, was collected with a Pasteur pipette and emulsified with collagen gelatin using a glass slide (Figure 2A, 2B and C). The material was divided into three parts and each part was distributed in gauze folded into six layers (Figure 2D), covered with the stool collector lid and subjected to the Rugai method for the second time (Figures 2E, F and G), in room temperature, with results evaluated after 2 and 4 hours. For the second stage, the procedure was repeated with the pellet formed from the first Rugai after another 2 hours.

The larvae (Figure 2H) obtained at the end of the two stages were analyzed for their purity, washed three times with dechlorinated water by centrifugation, to completely remove the collagen, and frozen at -20ºC for further research.

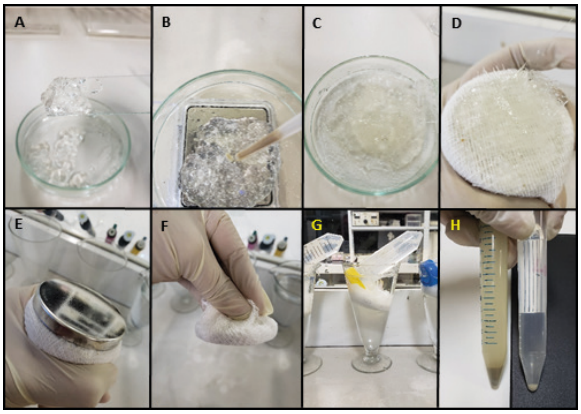

Figure 2: Purification of larvae with collagen. A: emulsified collagen; B and C: mixture of Rugai product and collagen; D, E, F and G: Rugai with collagen; H: difference between Rugai products without and with collagen. Source: Melo, 2019.

RESULTS AND DISCUSSION

The rodent feces used corresponded to 39g, generating approximately 46,400 first-stage larvae without the use of collagen and, from these, 27,650 purified with protein gelatin. In this sense, the recovery rate corresponded to 59.59%.

In the first stage, 23,200 larvae were recovered from the 2-hour Rugai. Of these, 6,250 were purified after 2 hours of Rugai with collagen and 3,200 in 4 hours (Figure 3).

Rugai Method	First Step		
	2h	4h	Total
No collagen	23,200	-	23,200
With collagen	6,250	3,200	9,450
Recovery rate (%)	26.94	13.79	40.73

Figure 3: Obtaining larvae before and after the purification process with semi-hydrolyzed collagen in the first stage.

In the second stage, the 4-hour Rugai yielded an additional 27,500 L1, with 10,950 in the first 2 hours of purification and 7,250 after 4 hours (Figure 4).

Rugai Method	Second Step		
	2h	4h	Total
No collagen	-	27.500	27.500
With collagen	10,950	7,250	18,200
Recovery rate (%)	39.82	26.36	66.18

Figure 4: Obtaining larvae before and after the purification process with semi-hydrolyzed collagen in the second stage

Regarding the presence of particles together with the larvae recovered from the samples, before and after the purification process, Figure 5 schematically presents a subjective scale of dirtiness. Therefore, Rugai without collagen, regardless of the observation time, presented grade A (very dirty). On the other hand, the product with collagen obtained from the first stage, in 2 hours, showed no signs of dirt, being classified as grade B (pure).

The same did not occur with the sample recovered after 4 hours, which showed grade C (rare particles). Finally, the samples from the second stage showed grades D and E, in 2 and 4 hours respectively.

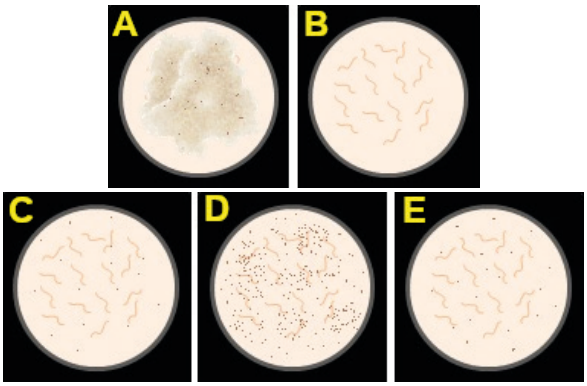

Figure 5: Scheme of subjective classification of dirt levels presented in the L1 samples recovered at the end of each stage. A: very dirty; B: pure (without dirt); C: rare particles; D: moderate; E: few particles. Source: Melo, 2019.

Figure 6 exemplifies the results of the procedures up to the second hour of the first stage, with collagen, at three different moments. The first indicates the fresh form of the sample and the concentration of material at magnifications of 16, 100 and 200 times. In this state, it is almost impossible to distinguish the larvae at all magnifications. However, structures of different sizes and shapes can be observed. In the second moment, although the structures are smaller, the quantity still does not allow for easy observation of larvae. In the third phase, 2 hours after using the semi-hydrolyzed collagen, at 16x magnification it is clear that the shapes are not identifiable, but are homogeneous. At 100x magnification, these forms are already perceived as first stage larvae and, at 200x magnification, it is not possible to identify substances other than the larvae in the sample.

These results indicate that, even with the loss of 76.06% of mining, the degree of purification is quite promising when the main objective is the quality of the material to be used.

The process also showed that extending the Rugai time with collagen significantly increases the number of larvae recovered, but other particles begin to appear as the collagen dissolves in the water. This suggests that, while the collagen remains in its gelatinous form, it offers physical resistance to inanimate particles, while the larvae are able to move and escape the sticky substance, sinking freely to the bottom of the calyx.

CONCLUSION

Semi-hydrolyzed collagen showed varying efficiency in the L1 purification process in rodent feces, depending on the time and amount of sediment obtained from the initial Rugai. Therefore, the shorter the exposure time, the higher the degree of purity and the lower the number of larvae recovered. Thus, when the objective is to obtain samples with a high degree of purity, the process is justified. This study also raises the possibility of purifying larvae in samples from other animals, as well as humans.

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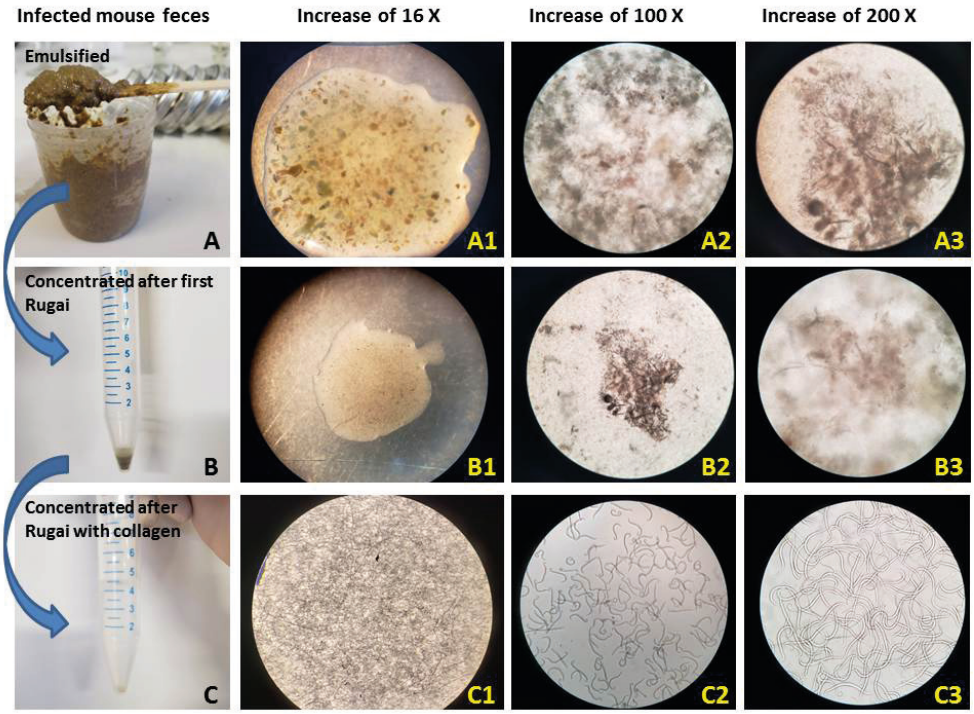

Figure 6: Macroscopic (A, B and C) and microscopic appearance (A1 to A3, B1 to B3 and C1 to C3) of feces from Wistar rats with L1 of *A. cantonensis*. A: fresh and emulsified samples with the addition of dechlorinated water. B: sample after Rugai without collagen; C: sediment after purification with semi-hydrolyzed collagen: Source: Melo, 2019

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