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Original Article

Development of a Double Glass Mounting Method Using Formaldehyde Alcohol Azocarmine Lactophenol (FAAL) and its Evaluation for Permanent Mounting of Small Nematodes

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Abstract

Background: Permanent slide preparation of nematodes especially small ones is time consuming, difficult and they become scarios margins. Regarding this problem, a modified double glass mounting method was developed and compared with classic method.

Methods: A total of 209 nematode samples from human and animal origin were fixed and stained with Formaldehyde Alcohol Azocarmine Lactophenol (FAAL) followed by double glass mounting and classic dehydration method using Canada balsam as their mounting media. The slides were evaluated in different dates and times, more than four years. Different photos were made with different magnification during the evaluation time.

Results: The double glass mounting method was stable during this time and comparable with classic method. There were no changes in morphologic structures of nematodes using double glass mounting method with well-defined and clear differentiation between different organs of nematodes in this method.

Conclusion: Using this method is cost effective and fast for mounting of small nematodes comparing to classic method.

Introduction

Nematodes are one of the most important human parasitic diseases agents. They are divided into three

types; parasitic, zoonotic and free living agents. Usually parasitic and zoonoses cause disease in human (1-3). However, in some cir-

cumstances free-living nematodes may also cause disease in human (4). Accordingly, study on all aspects of nematodes specially those related to their morphology is necessary for better understanding of these organisms. Basic knowledge for study of nematodes is based on their morphology and their related life cycle. In this regard, it is very important to have good specimens with fine details. Students learn about morphology of nematodes or their pathological effects in practical sessions along with theoretical courses. This causes better understanding of related diseases and subsequently better achievements in the control and treatment of nematode parasites. It is obvious that without good quality specimens in teaching laboratories, students cannot be trained well.

Re-emerging of the eradicated helminthes makes us serious to collect and mount specimens as much as possible in high quality condition to be taught to the new students, who are not familiar with the eradicated parasites. Therefore, lack of these samples for teaching to medical and allied sciences will result misunderstanding and misdiagnosis in their future career. For many years, the scientists have tried to prepare permanent slides (5-9). Recently, a few staining and mounting protocols including the application of red beet extract on the staining and differentiation of the helminth organs have been used for better differentiation between initial organs of worms (10). The application of different methods for staining different type of helminthes is necessary in order to laid in classification and identical key (10). Other chemicals such as silver staining have been used for elucidation of the synloph in Trichostrongyle nematodes (11). The classic way of mounting using Canada balsam with dehydration of the worms needs a critical work and special attention during worm processing, otherwise, it will destroy the worm (12).

It is very important to have good knowledge and information about fixatives

and chemicals that are used for preserving the samples; as the samples may be preserved for several purposes. For example if we decide to determine both the morphological and molecular aspects of nematode, high concentration of alcohol is not suitable and causes changes in organs measurements which itself could be used for numerical taxonomy (13).

Among parasite helminthes, nematodes (coelomates) especially the small ones are difficult to be dehydrated during staining and mounting. Sometimes the prepared slides will be black and destroyed; so, not suitable for teaching purposes.

Present study was carried out with the aim of preparing high quality permanent slides from small nematodes using Formaldehyde Alcohol Azocarmine Lactophenol (FAAL) as their fixing, staining and preserving media using a double glass method for better teaching parasitology specially taxonomy of nematodes which itself is very important to human related diseases. Classic mounting using dehydration of the specimens was used to evaluate and compare them in different dates for a long time to find which one is superior for mounting of small nematodes.

Material and Methods

Different nematode specimens including 20 species such as *Rhabditis* sp., *Strongyloides stercoralis*, *Nematodirus abnormalis*, *Marshallagia marshalli*, *Oestertagia circumcincta*, *Ostertagia* spp., *Haemonchus contortus*, *Necator americanus*, *Enterobius vermicularis*, *Syphacia obvelata*, *Aspicularis tritraptera*, *Trichuris* spp., *Parabronema* spp., *Trichostrongylus* spp. *Physaloptera* sp. and *Toxocara cati* from different sources including field investigations and abattoir surveys were collected. The specimens were washed and finally worm samples were transferred into normal saline followed by fixing in 10% formaldehyde (Merck, Germany) and/or 70% alcohol

(Merck, Germany). The specimens were transferred into FAAL, which clear, and stains the specimens (14-16).

Samples after recognition (17) were divided into two groups to be mounted in different ways: the first group was mounted by Canada balsam (Sigma-Aldrich, USA) using double glass mounting while the specimens were transferred into a Glycerin Jelly (GJ) medium. In this regard, the stained samples with FAAL, were transferred carefully into a suitable amount of GJ on the middle of a slide and covered with a small coverslip. After 3-5 minutes when GJ was gelated, the first coverslip was covered with a bigger coverslip while suitable amount of Canada balsam had been poured onto the first coverslip. The second group, were stained with Azocarmine followed by dehydration and using Canada balsam in a classic way (18). Briefly, all samples were passed through gradient dilution of alcohols, 70%, 80%, 90%, 96% and 100%, respectively. This step was followed by clearing with xylene- alcohol and pure xylene. Every step was repeated 2 times each time 5-10 minutes followed by transferring the samples into Canada balsam and covered with a coverslip.

The mounted specimens were carefully observed for their changes in cuticle and other organs for several months and were photographed using an Olympus microscope (CH-2, Japan) equipped with camera. The specimens were also photographed using Phase Contrast condenser.

Results

A total of 172 small nematodes including males, females and larvae were mounted using double glass mounting method in which FAAL was used as staining and clearing media. The smallest one was 250 μm and the largest one was about 30 mm. A total of 37 samples were mounted with classic method and compared with the specimens of double

glass mounting method.

The color of the most specimens using double glass mounting method were nearly light brown to pink (Figs.1-4) and their quality were high with very good differentiation between their organs especially reproductive organs (Figs. 5-7). No changes were observed during the time of observations.

Many slides, which prepared in classic method, have very good quality (Figs. 8-9). A number of these slides became black immediately after mounting (Fig. 10).



Fig. 1: The copulatory bursa of male *Necator americanus*, double glass mounting



Fig. 2: The male of *Necator americanus* double glass mounting. Phase contrast photo

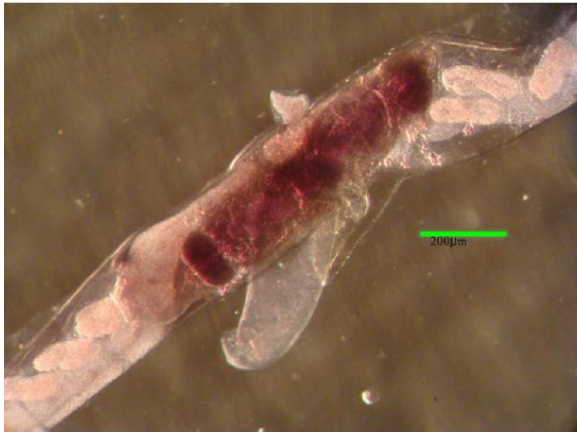


Fig. 3: The ojector of *Marshallagia marshali*, double glass mounting after 4 years

So it was necessary to transfer back them to xylene or even serials of alcohols to repeat dehydration again. In spite of doing this long time procedure, some of those samples remain black (Fig. 10).

Discussion

The impact of the methods for processing including killing, fixing, staining and mounting on some species of nematodes has been studied (18, 19).



Fig. 4: The whole mount (WM) of male *Strongyloides stercoralis* in double glass mounting



Fig. 5: The head of *Enterobius vermicularis* mounted in double glass method



Fig. 8: A slide including two *Rhabditis axei* male worm mounted with classic method, Canada balsam after 3 years

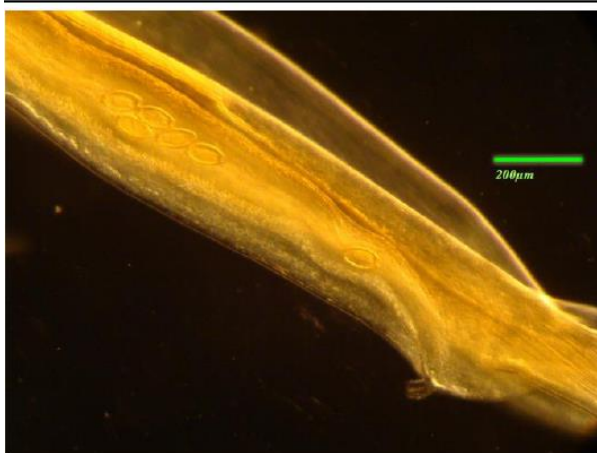


Fig. 6: The vulva of a female *Trichuris trichiura* mounted in double glass method



Fig. 9: A single glass mounted male of *Trichuris trichiura* after three years



Fig. 7: A female *Rhabditis axei* mounted in double glass mounting



Fig. 10: A slide including two *Rhabditis axei* male worm mounted with classic method, Canada balsam after 3 months

Since the process of preparing a slide, exposes a worm to many chemicals, pressures and temperature changes that has effect on their taxonomic characters, choosing a perfect method is very important. Grewal et al. compared the effect of several methods for killing, fixing and mounting of the *Coenorhabditis elegans* on its taxonomic characters. They found TAF fixative (7 ml 40% formaldehyde, 2 ml triethanolamine, 91 ml distilled water) at 95 °C and also slow transfer of the nematode from fixative to glycine as mountant has minimum effect on shrinkage of nematode (19). In addition, they tried to prepare a special agar con-

taining CuSo₄ as a cover glass supporter. This slide was temporary slide but when this slide was sealed with Thornes cement (Zut or Glyceel), nail varnish or Araldite resin (7) a permanent mounted of slide was made (20). In our work, double glass mounting using FAAL prevents shrinkage of the nematodes that usually take place in dehydration procedure methods. This could be observed obviously in mounted slides (Figs. 1-6). On the other hand, the double glass mounting method was stable during a long time following mounting. Moreover, there were no changes in morphologic structures of nematodes using double glass

mounting method. Taxonomic identification is very time consuming and might require the submission of specimens to an expert. In the survey, Sepulveda and Kinsella fixed the helminths which were obtained from the wild animals (either in 70% ethanol, 10% buffered formalin, or alcohol-formalin-acetic acid) and for species identification, they cleared nematodes and small acanthocephalans in lactophenol and stained trematodes, cestodes, and large acanthocephalans, using Harris' hematoxylin or Semichon's carmine. Then they recognized the species by examining different structures (e.g. male spicules in nematodes or the rostellum in cestodes) (21). In all of the works differentiation of organs are very important. Comparing to other works, our results showed that the double glass mounting revealed well-defined and clear differentiation between different organs of nematodes in this method.

Ryss reported a technique to prepare permanent slides of nematodes in which the living nematode was put in hot formaldehyde, and then it was transferred into glycerin to be mounted, during a definite process. A drop of glycerin containing nematode was occupied in the hole of slide. Two pieces of waxed paraffin were put on both sides of the hole. A cover glass was put on the top of hole and paraffin. Then it was fixed on the sample by means of heater. All of internal and external structures were fine (22). In our work, this kind of slide was also used. Lack of using the paraffin and heat causes a big bubble. Kumagai et al. had the experience like Ryss for helminth eggs. There were some disadvantages with their slides. One of them was related to use of Glycerin-Jelly, which has low melting point, without Canada balsam, so they must be kept away from heat. In addition, an objective lens of 100× magnification cannot be used for them because eggs are in the hole of the slide and are away from cover glass (23). It seems that the problem of Ryss and Kumagai slides are the same. Anyway, in our experience, Glycerin-

Jelly was used and protected by Canada balsam, in a double glass mounting method.

In spite of the traditional method that is difficult to mount the small worms, the double glass is easier. Moreover, the color of the slides in double glass yields a better differentiation between different organs in the worm especially when they are observed with Phase Contrast condenser (Figs. 3, 4 and 8-11). Reproduction and digestive systems are set in the pseudo-coelom of nematodes. Every part of these two systems has especial histologic tissues. This kind of preparing and mounting slides is very useful for determining and comparing of these tissues in a nematode and/or between several nematodes.

Anyway, this experience showed that the classic method for preparation slides from small nematodes like, *Strongyloides stercoralis* or *Rhabditis axei*, face to a plenty of problems so that most of them are going to have shrinkage or black, while the mentioned problems for bigger nematodes are moderate.

The most important item for double glass method is recovering the nematodes in different angles. They can be used for viewing their specific structure using specific condensers such as Nomarski and Phase Contrast (24, 25). Our results showed a very interesting photos using Phase Contrast condenser for viewing double glass mounted nematodes. Therefore, double glass mounting could be one of the best methods to prepare permanent slides for small nematodes. Using this method is cost effective and fast for mounting of small nematodes comparing to classic method.

Conclusion

In comparison between two methods of single and double glass mounting the following are the advantages of double glass mounting:

1. Saving time, since there is no need dehydration process.

2. Saving samples for small nematodes and larvae, since these fine nematodes are going to be missed during the process of dehydration.

3. Saving the money, because there is not any need to use Alcohols and xylene.

4. Three-dimensional investigation of samples, despite of being permanent slides, changing the coverslips is very simple and also hydrated samples are flexible, so that if it is necessary we can change the samples figure.

5. Solving the blackness problem during dehydration due to their pseudo-coelom.

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