Effects of Fixation and Dehydration Procedures on Marine Nematodes

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Abstract: Different combinations of fixation and dehydration procedures for the preparation of permanent mounts of marine nematodes of the subfamily Oncholaiminae were tested and compared. Qualitatively, the best specimens resulted from Seinhorst's killing method and fixation in FAA; the dehydration procedure was of less significance. Quantitatively, no significant modification of measurements resulted from any of the methods used. Sources of error in measurements are discussed.

Although many qualitative observations of effects of fixation and dehydration on nematodes have been published, few of these have dealt with dimensional changes resulting from these procedures. Lamberti (2) studied the changes in the taxonomically important measurements of *Longidorus africanus* produced by various methods of preparation.

MATERIALS AND METHODS

Preliminary qualitative observations of fixative and dehydration effects were made using *Adoncholaimus crassicaudus* Wieser (collected from *Enteromorpha* sp., Bodega Bay, California) and *Oncholaimium* sp. from Puget Sound, Washington. These nematodes were killed by gradual heating (60 C for 15 min) and exposed to the following fixatives for at least 48 hr: (i) 2½% formalin (room temperature) in distilled water, (ii) 5% formalin in distilled water, (iii) 2½% formalin in sea water, (iv) 5% formalin in sea water, (v) 2½% warm formalin (60 C) in sea water, (vi) 5% warm formalin (60 C) in sea water, (vii) warm FAA (60 C) in sea water, and (viii) warm TAF (60 C) in sea water. Nine specimens from each fixation treatment were processed in glycerine: three by Baker's rapid method (1), three by the methanol method (6) and three by the slow method (1). No measurements were made on any of these specimens.

Measurements as well as qualitative observations were made on *Oncholaimus apostematus* Wieser from Miller Park, Tomales Bay, California. Groups of 10 nematodes each were processed by the following combinations: (i) 2½% warm formalin (60 C) in sea water + Baker's method, (ii) 5% warm formalin (60 C) in sea water + methanol method, (iii) 2½% hot formalin (90 C) in distilled water + Baker's method, (iv) Seinhorst's method + FAA + methanol method, (v) Seinhorst's method + TAF + slow method, (vi) Seinhorst's method + 2½% formalin (room temperature) + Baker's method, (vii) vapor-phase perfusion (3) + TAF + slow method, (viii) vapor-phase perfusion + FAA + slow method, (ix) vapor-phase perfusion + 2½% formalin (room temperature) + methanol method, and (x) hot TAF (90 C) + methanol method.

The length of esophagus, esophagus to anus, tail, and the maximum and anal body diameters were determined for each specimen under a cover glass supported by hairs with the aid of an ocular micrometer. The hanging drop method of Lamberti (2), using fresh egg white, was found unsuitable for slowing down the nematodes for measurement. Moreover, it was found that a magnifying effect up to 25% occurs simply by enlarging the size of the hanging drop.

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Natural dimensions were obtained for comparative purposes in groups i–iii and x using quiescent or moribund specimens after all-night chilling at 4 C; these were later killed by the fixative. Active living nematodes were used for fixation by Seinhorst's method (iv–vi) and the vapor-phase perfusion method (vii–ix); these were measured for the first time after fixation.

The methanol method of dehydration employed was that of Wieser and Hopper (6), using 10% glycerin in methanol at 54 C. The solution was placed in a BPI watch glass in a petri dish and warmed up to 54 C; the nematodes were transferred to the methanol one by one as it remained in the oven at constant temperature. The specimens were kept in glycerin in a desiccator overnight before mounting, as were the specimens dehydrated by Baker's rapid method and the slow glycerin method. The slow method utilized 2½% glycerin in distilled water for 24 hr, followed by transfer to 5% glycerin and evaporation for five or six weeks in a petri dish with a wet filter paper disc on the bottom.

RESULTS

ESTIMATION OF SOURCES OF MEASUREMENT ERROR: It seemed meaningless to establish the effects of physical and chemical treatments on the measurements of nematodes without knowing the normal variations which can occur in measuring. Reliability of measurement was checked from two viewpoints: operator technique and mechanical and optical imperfections.

Operator Technique.—Do measurements of the same specimens vary when taken at different times by the same person, using the same measuring apparatus? To test this, the lengths of 10 specimens of *Oncholaimus apostematus* were measured three separate times with a monocular compound microscope and ocular micrometer. The highest average of the 10 measurements was found to be 2.8% greater than the lowest, with one individual specimen varying up to 14%.

Do measurements of the same specimens vary when measured on different microscopes or by different individuals? To test this, we asked Dr. Armand Maggenti and Dr. Robert Kinloch to measure (three separate times) the same 10 specimens of *Oncholaimus* used in the previous test. Both used Leitz Ortholux microscopes and a large camera lucida arrangement of the type recommended by Thorne (5). The calibration charts were those in standard use with each microscope. The average of one operator's measurements was from 0.75 to 3.5% greater than the averages of the three previous measurements, while the average of the other operator was 4.2–7% greater.

Mechanical and Optical Imperfections.—It was suspected that the large first surface mirror of the camera lucida might distort measurements toward the margins. To test this, two fixed distances on a stage micrometer were compared to the scale of an ocular micrometer at extreme upper and lower, left and right margins of the field. Both low (100 ×) and high (400 ×) power of two Ortholux microscopes were checked in this way. The following averages are based upon three measurements taken at each position.

Microscope 1:	Low power—8.8% greater length at bottom margin than at top 2.9% greater length at left margin than at right High power—7.0% greater length at bottom margin than at top 1.5% greater length at left margin than at right
Microscope 2:	Low power—8.3% greater length at bottom margin than at top 2.1% greater length at left margin than at right High power—6.4% greater length at bottom margin than at top 0.4% greater length at left margin than at right

It can be seen from the above that the

position of a specimen on a slide and the length of the structures measured can introduce variation in measurement. These discrepancies probably are due to the difficulty in precisely aligning the mirror to the exact theoretical angle required for accuracy. Use of an ocular micrometer whenever possible will reduce or eliminate this potential source of error.

Qualitative Effects of Fixation and Dehydration Procedures.—Fixation is the chief determinant of the histological changes during preparation of permanently preserved specimens on slides. Dehydration procedures cannot correct distortions which have previously occurred. Although some good and even excellent specimens were obtained by all methods of fixation, in general it can be stated that warm fixatives were superior to cold fixatives and that 90 C gave better results than 60 C. Vapor-phase perfusion produced many specimens twisted or coiled in a full circle, whereas death in storage or death brought about by hot fixatives resulted in straight specimens. However, excellent specimens were obtained by perfusion followed by FAA fixation. TAF brought about excellent clearing but resulted in a somewhat glassy appearance. Seinhorst's killing method, followed by any of the three methods of dehydration, consistently gave the best results. Of the fixatives, FAA was by far the most satisfactory. The natural color of the intestine was preserved; body and organ contours, musculature and esophageal lining were straight; and the excretory pore and ampulla, nuclei and nucleoli and cell outlines were distinct. Hot 2½% or 5% formalin also gave natural-looking specimens. There was no observable difference between fixatives prepared with sea water and those prepared with distilled water.

Of the dehydration procedures tested, the most uniformly excellent results were achieved by the slow method. The methanol method sometimes produced variations in body or organ contours and specimens were occasionally shrunken through being stranded on the walls of the BPI dish due to turbulence. The Baker rapid method gave good results but requires more handling of specimens.

Quantitative Effects of Fixation and Dehydration Procedures.—Each nematode was measured after each procedure. The differences between the measurements within each of the procedural combinations were analyzed by the "t" test. This was considered to be more reliable than comparing the average of each test group against the average resulting from a standard method. Of the 10 combinations tested, none showed significant variation in measurements from the original measurements after fixation or dehydration.

Conclusions

Maggenti and Viglierchio (3) concluded that Seinhorst's heat-killing method, followed by formalin vapor or FAA treatment, produced the best specimens of soil nematodes. Lamberti (2) found Seinhorst's killing method followed by fixation with FAA the most satisfactory for *Longidorus africanus*. The results presented here show that the best preparative procedure for marine oncholaims is killing by Seinhorst's method, followed by FAA fixation. FAA also gave excellent results when used by itself as a killing agent or as a fixative following vapor-phase perfusion.

Our findings differ from those of Lamberti (2) in that no significant modification of measurements was brought about by any of the methods used. This difference may reflect the different method of analysis of the data or the fact that marine and soil nematodes differ in cuticular permeabilities. In

general, more delicate infiltration measures are required for soil or plant-parasitic nematodes than for most marine nematodes. It should be pointed out also that all three species used in this study were members of the subfamily Oncholaiminae, and the conclusions reached for them may not apply to all marine nematodes. In evaluating the various techniques qualitatively it should be remembered, as Maggenti and Viglierchio (3) remarked, that "the suitability of any specimen is determined by the use to which it will be put." Hence, specific procedures may be required for maximum definition of specific tissues or organs in a given specimen group.

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