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HEALTH PRODUCTS AND FOOD BRANCH

OTTAWA

ISOLATION AND IDENTIFICATION OF ANISAKID ROUNDWORM LARVAE IN FISH

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1. **APPLICATION**

These laboratory procedures are applicable to the isolation and identification of live third-stage anisakid roundworm parasites commonly found in the flesh or on the viscera of a variety of commercially important marine fishes. These procedures are meant for use by laboratory and inspection staff, and are intended to be used for small quantities of fish originating from consumer complaints, as well as for larger samples collected in routine inspections. This revised method replaces ExFLP-1, dated September 1995.

2. **PRINCIPLE**

The procedures involve manual, sedimentary, or chemical separation of roundworm larvae from fish tissues. They are either non-destructive (candling and ultraviolet illumination), or destructive (elution and digestion) techniques. Identification is based on gross morphological features which may be observed microscopically in live or preserved specimens. Candling is currently the only procedure used in routine inspections at federally regulated processing plants. Illumination with ultraviolet light is another laboratory method used for detecting anisakid larvae at, or near, the surface of the flesh or viscera by their fluorescence. Although it will kill any live parasites, detection through exposure to ultraviolet light is improved by a preliminary freeze/thawing of the specimen.

Elution involves the migration of some (but not all) live parasites into saline while, in digestion, all parasites, live or dead, are isolated. While no significant difference in the total number of worms recovered by either technique was observed, the numbers of pathogenic worms (eg. *Anisakis* and *Pseudoterranova*) recovered by the digestion method can be 175% higher than the number recovered by elution (10.2). As the parasite yield may be improved somewhat, a combination of candling, elution, and digestion is recommended.

3. **GENERAL CHARACTERISTICS OF ANISAKID ROUNDWORMS**

3.1 Life Cycle

The anisakid roundworms include a number of closely related genera within the phylum Nematoda, with a relatively complex life cycle (Fig. 1) involving a free-living stage and multiple hosts. Eggs released from the

mature worms are passed in the faeces of marine mammals, which act as the definitive hosts. The eggs sink to the sea floor and hatch into second-stage larvae within days or weeks depending upon the water temperature. These larvae then rely upon ingestion by marine crustaceans in order to facilitate their continued development into the third-stage. When the crustacean is eaten by a fish or squid the larvae migrate into the tissues of this second intermediate host and develop to the advanced third-stage on the viscera or in the muscle. When an infected fish is eaten by a definitive host such as a marine mammal, the larvae are released into the stomach or intestine where they undergo further moults, developing into fourth-stage larvae and eventually adults. Humans can only be considered accidental hosts in this life cycle, and have no influence on the transmission of these parasites.

3.2 Larvae of Public Health Importance

The larvae of two anisakid roundworms, *Anisakis simplex* (herring worm), and *Pseudoterranova decipiens* (seal worm), have been implicated in the majority of cases of human anisakiasis. The infective larval stage may be found on the viscera or in the flesh of a number of commercially important fish species, including salmon, cod, herring, and mackerel, as well as squid (10.1). *P. decipiens* is also found in smelt and various flatfish. Two other anisakid roundworms, *Contracaecum osculatum* and *Phocascaris* spp., have also been identified in commercially important marine fishes and may be of public health concern.

4. MATERIALS AND SPECIAL EQUIPMENT

4.1 Candling

- 4.1.1 candling light table as described below
- 4.1.2 small forceps and culture dish containing normal saline (0.85 g NaCl per 100 mL distilled water)

4.2 Ultraviolet Light Illumination

- 4.2.1 hand-held or fixed ultraviolet light illuminator
- 4.2.2 face-shield or goggles (UV light resistant)
- 4.2.3 small forceps and culture dish containing normal saline

4.3 Elution

- 4.3.1 U.S. Standard No. 4 sieve (or similar sized kitchen sieve)
- 4.3.2 large funnel (500 mL or larger) or other vessel
- 4.3.3 clear, flexible tubing and tubing clamp
- 4.3.4 ring clamp with support rod and base
- 4.3.5 normal saline
- 4.3.6 plastic wrap or aluminum foil
- 4.3.7 250 mL beaker
- 4.3.8 stereoscopic microscope or magnifier
- 4.3.9 forceps and culture dish containing normal saline

4.4 Digestion

- 4.4.1 pepsin solution [15 g pepsin (1:10,000; Sigma Chemical Co.) dissolved in 750 mL normal saline]
- 4.4.2 1.5 L beaker, 250 mL beaker
- 4.4.3 shaking water bath (37°C), or bath with mechanical stirrer
- 4.4.4 pH meter, or pH paper, and 6 N HCl
- 4.4.5 aluminum foil
- 4.4.6 U.S. Standard No. 18 sieve (or similar sized kitchen sieve)
- 4.4.7 large container to hold the sieve for collection of sieved material, or US Standard No. 140 sieve
- 4.4.8 normal saline to rinse sieve

- 4.4.9 funnel apparatus as described for elution
- 4.4.10 stereoscopic microscope or magnifier
- 4.4.11 forceps and culture dish containing normal saline

4.5 Preservation and Storage

- 4.5.1 fixatives:
glacial acetic acid, or alcohol-formalin-acetic acid (AFA) (85 mL 85% ethanol, 10 mL formalin, 5 mL glacial acetic acid)
- 4.5.2 preservatives:
70% ethanol, or alcohol-glycerol (9 parts 70% ethanol, 1 part glycerol)
- 4.5.3 clearing agents:
glacial acetic acid, or glycerol, or xylene

5. DETECTION AND ISOLATION PROCEDURES

5.1 Candling

5.1.1 Candling light table

The recommended light table for candling should have at least two 20 watt "cool white" fluorescent tubes (10.5). The light source should be held by a rigid framework below a white, translucent acrylic plastic or other suitable material with a translucency of between 45-60%. This working surface should be approximately 30 x 60 cm and 5-6 mm in thickness. The average light intensity should be between 1500 and 1800 lux as measured 30 cm above the center of the acrylic sheet. Overhead illumination should be at least 500 lux. Adequate results may also be obtained in the laboratory using a simple photographic light table.

Candling works equally well with fresh or previously frozen fillets. The efficiency of the candling technique is largely dependent upon the thickness of the fillet being examined (10.5). Large, thick fillets, therefore, may pose a particular problem due to the increased production costs and loss of value involved with slicing such fillets. The candling technique is generally considered to be a costly and inefficient means of detecting and removing parasites from fillets. The technique is also limited in that it cannot distinguish between live and dead parasites.

- 5.1.2 Place fillet on a light table and examine for the presence of tightly coiled, encapsulated larvae, which will appear as dark spots. Unencapsulated larvae may also be observed on the surface of the fillet.

- 5.1.3 Use forceps to transfer any visible worms to a dish of normal saline.

5.2 Ultraviolet Light Illumination

- 5.2.1 Wear an ultraviolet resistant face shield or safety goggles.

- 5.2.2 In a darkened room, hold UV light approximately 10 cm above surface of fillet and examine for fluorescent worms. The colour of the fluorescence aids in a preliminary identification. *Anisakis* and *Pseudoterranova* larvae fluoresce a bright bluish-white colour while *Contracaecum* larvae fluoresce yellow (10.4). As with candling, this procedure will not distinguish between live and dead parasites.

- 5.2.3 Use forceps to transfer worms to a dish of normal saline.

5.3 Elution

- 5.3.1 Transfer up to 200 g of fish tissue to a U.S. Standard No. 4 sieve (or equivalent sized kitchen sieve) positioned in a large funnel or other vessel. Attach tubing to the neck of the funnel and close off tightly with a tubing clamp.
- 5.3.2 Fill funnel or vessel with normal saline (completely immersing fish tissue). Cover with plastic wrap or aluminum foil and leave apparatus overnight at room temperature. Live larvae will migrate out of the tissues and fall through the sieve and into the neck of the funnel (or into the bottom of the vessel).
- 5.3.3 After 16 to 18 h, drain 100 mL of sediment into a beaker. Using forceps, remove any visible worms to a culture dish containing normal saline and carefully examine the remaining suspension, using a stereoscopic microscope (120x) or magnifier, for any smaller worms.

5.4 Digestion

- 5.4.1 Add about 200 g of fish tissue to 750 mL of 37°C pepsin solution in a large beaker.
- 5.4.2 Place the beaker in a 37°C shaking water bath so that the level of the water is within 1 cm of the fluid level in the beaker. Shake the sample at low speed for about 15 min.
- 5.4.3 Adjust the sample to pH 2 with 6 N HCl, cover beaker with foil, and continue shaking for 24 h (or until the tissue is fully digested).
- 5.4.4 Pour the digested material through a U.S. Standard No. 18 sieve (or equivalent sized kitchen sieve) into a suitable container. Rinse the sieve with normal saline and examine the washed remains by immersing the mesh in clean normal saline. Use forceps to transfer worms to a culture dish containing normal saline.
- 5.4.5 Examine the material which passed through the sieve by transferring it to a clamped funnel, allowing it to settle for 1 h, and draining the sediment into a beaker as described for elution. Examine for smaller worms using a stereoscopic microscope (120x) or magnifier.
- 5.4.6 Alternatively, digested material can be passed through nested No. 18 and No. 140 U.S. Standard sieves, which are then separated, and the screen portion of the sieves immersed in normal saline. Collect and transfer to a dish of normal saline any of the larger worms caught in the No. 18 sieve. Similarly, transfer any smaller worms caught in the No. 140 sieve to normal saline.

6. **RECORDING RESULTS**

All parasites isolated from a sample should be recorded as either live, dead (intact), or fragments. Parasites may be considered live if they demonstrate any movement during the isolation procedures. Anisakid larvae generally become much more active as they approach room temperature.

In addition to numbers of parasites present per kg of product, a preliminary identification of these parasites (or a brief description) should be included if possible. The species of fish examined, the source, the total sample weight, and the weight and number of individual samples (fillets) should also be recorded, along with any other relevant information.

7. **PRESERVATION AND STORAGE OF LARVAE**

A variety of preservation techniques have been used for nematodes. Live or previously frozen worms should first be fixed in glacial acetic acid or AFA (1 min) , and then preserved in small glass vials containing 70% ethanol, or, preferably, an alcohol-glycerol solution. If necessary, worms may be stored in this solution indefinitely. Vials should be adequately labelled (eg. parasite identification, host species, location in host, sample number, date of recovery, name of examiner, etc.)_

8. PARASITE IDENTIFICATION

For microscopic examination, the use of a clearing agent such as xylene may be necessary to make the nematode cuticle more transparent. A temporary slide mount can be prepared, after which the worms are returned to the preservative solution. Nematodes are not generally stained for microscopic examination.

Third-stage larvae of *A. simplex* can generally be distinguished from *P. decipiens* on the basis of their size and colour. A positive identification can be made by examining the anterior gut structure (10.3) which is easily seen in fresh or preserved larvae using a stereoscopic microscope (Fig.2). Third-stage larvae of *Anisakis simplex* are small white worms, 9-36 mm in length, with a straight anterior gut structure consisting of esophagus, ventriculus, and intestine. *P. decipiens* are typically reddish-brown in colour, 9-58 mm in length, and have an anteriorly projecting intestinal caecum. *Contracaecum* and *Phocascaris* third-stage larvae are very difficult to distinguish morphologically and are often discussed as a single group. Third-stage larvae within the *Contracaecum/Phocascaris* complex can, however, be readily distinguished from those of *A. simplex* and *P. decipiens*. The former are greenish-brown in colour and 7 to 30 mm in length, with both anteriorly projecting intestinal caecum and posteriorly projecting ventricular appendix (10.3).

9. INTERPRETATION

If eaten raw or undercooked, fresh fillets containing any anisakid roundworm larvae may be considered a health hazard. There is currently no HPFB guideline for parasites in fish. However, guidelines have been established by other regulatory bodies in Canada and should be consulted. Tolerance limits are not included here as they are subject to change.

If there is a question as to the acceptability of a lot, contact: Evaluation Division, Bureau of Microbial Hazards, Food Directorate, Health Products and Food Branch, Health Canada, Telephone (613) 957-0349 or FAX (613) 952-6400.

10. REFERENCES

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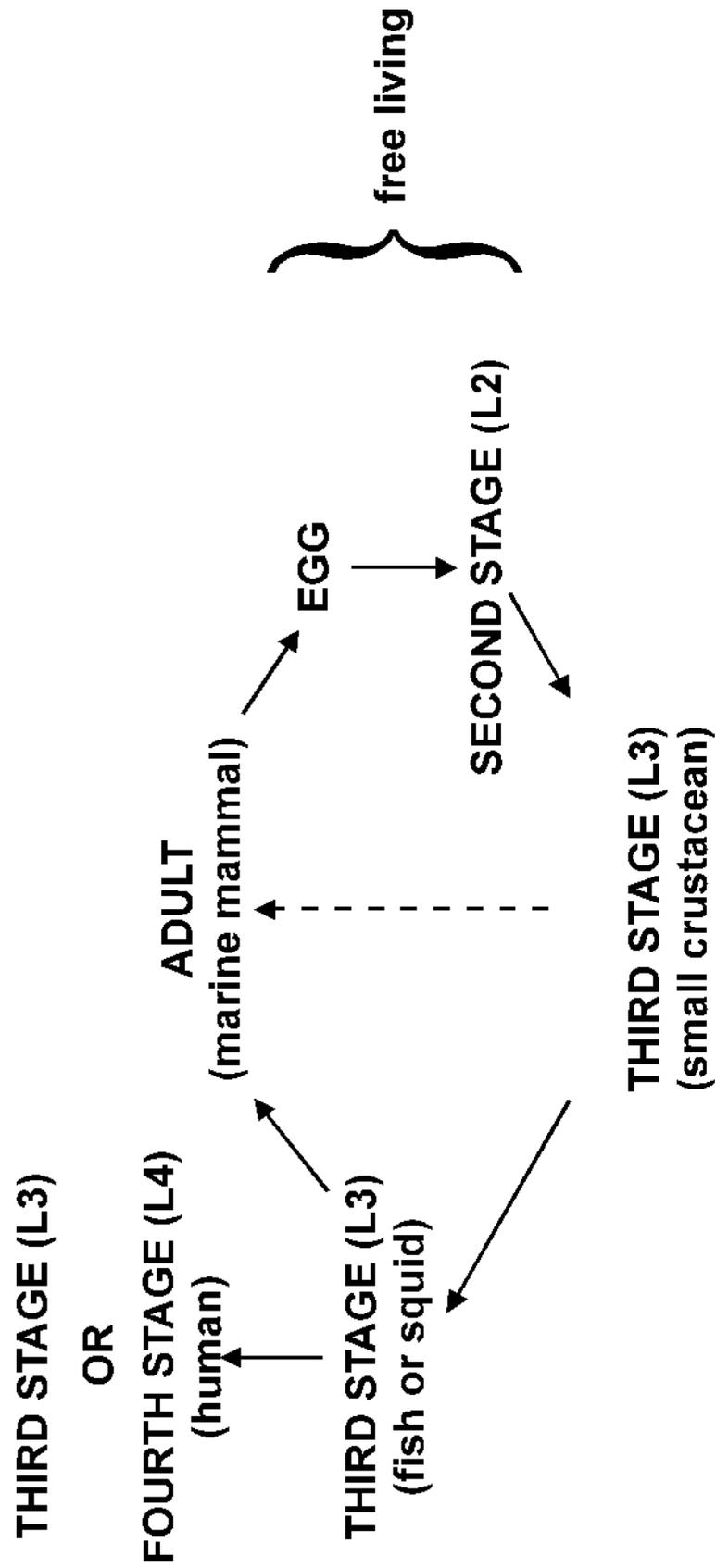


Figure 1. Generalized life cycle of anisakid roundworms

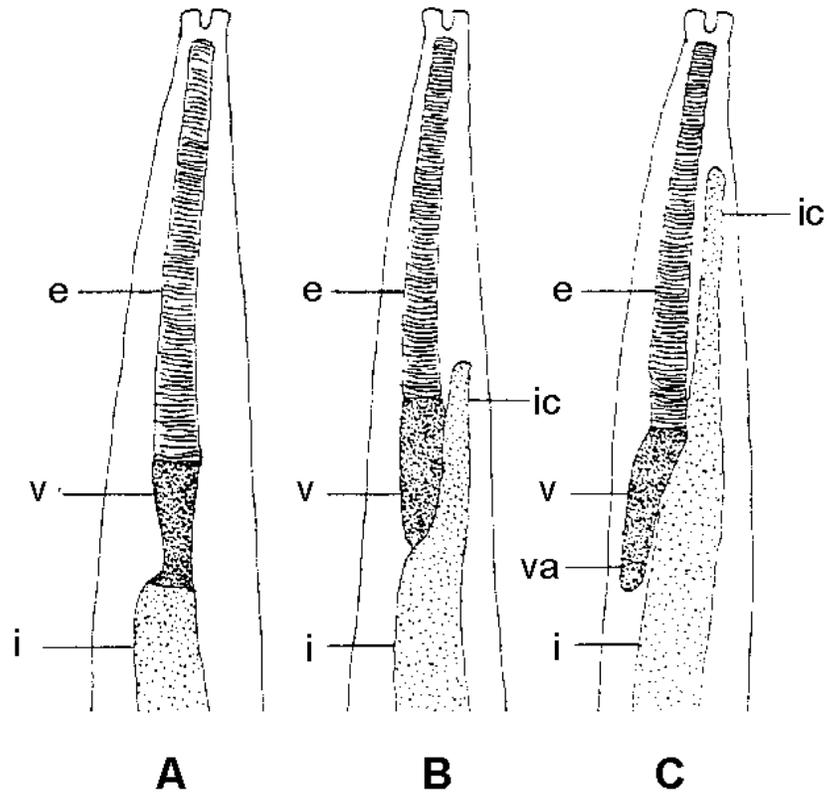


Figure 2. Anterior end of third stage anisakid larvae. A. *Anisakis simplex*; B. *Pseudoterranova decipiens*; C. *Contracaecum/Phocascaris* complex. e esophagus, v ventriculus, i intestine, ic intestinal caecum, va ventricular appendix. (From Olson et al. 1983).