Video Article

Helminth Collection and Identification from Wildlife

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URL: http://www.jove.com/video/51000
DOI: doi:10.3791/51000

Keywords: Environmental Sciences, Issue 82, Helminths, eukaryotic parasites, worms, nematodes, cestodes, trematodes, acanthocephalans, wildlife

Date Published: 12/14/2013

Citation: Sepulveda, M.S., Kinsella, J.M. Helminth Collection and Identification from Wildlife. *J. Vis. Exp.* (82), e51000, doi:10.3791/51000 (2013).

Abstract

Wild animals are commonly parasitized by a wide range of helminths. The four major types of helminths are "roundworms" (nematodes), "thorny-headed worms" (acanthocephalans), "flukes" (trematodes), and "tapeworms" (cestodes). The optimum method for collecting helminths is to examine a host that has been dead less than 4-6 hr since most helminths will still be alive. A thorough necropsy should be conducted and all major organs examined. Organs are washed over a 106 µm sieve under running water and contents examined under a stereo microscope. All helminths are counted and a representative number are fixed (either in 70% ethanol, 10% buffered formalin, or alcohol-formalin-acetic acid). For species identification, helminths are either cleared in lactophenol (nematodes and small acanthocephalans) or stained (trematodes, cestodes, and large acanthocephalans) using Harris' hematoxylin or Semichon's carmine. Helminths are keyed to species by examining different structures (*e.g.* male spicules in nematodes or the rostellum in cestodes). The protocols outlined here can be applied to any vertebrate animal. They require some expertise on recognizing the different organs and being able to differentiate helminths from other tissue debris or gut contents. Collection, preservation, and staining are straightforward techniques that require minimal equipment and reagents. Taxonomic identification, especially to species, can be very time consuming and might require the submission of specimens to an expert or DNA analysis.

Video Link

The video component of this article can be found at http://www.jove.com/video/51000/

Introduction

Vertebrates are parasitized by four major groups of helminths (worms). Two of the groups, trematodes, or flukes, and cestodes, or tapeworms, fall within the Phylum Platyhelminthes. The other two groups are the nematodes, or roundworms, (Nematoda) and the acanthocephalans, or thorny-headed worms (Acanthocephala). Many of these parasites have been documented as causes of morbidity and mortality in wild birds and mammals[1,2].

Most helminths have complex life cycles involving more than one host[1,2] . For instance, trematodes have one or two intermediate hosts (usually invertebrates) and a final host. All hosts need to be available for the cycle to be completed and thus for adult helminths to be present in the final hosts (which are the topic of this manuscript). So it is important to keep in mind that seasonal fluctuations can occur in the prevalence and intensity of some helminth species and long-term monitoring is desirable to capture the complete helminth of auna of a particular host.

The optimum method for collecting helminths is to examine a host that has been euthanized. This allows for the collection of helminths while they are still alive and for their proper relaxation and fixation. Material from hosts that have been dead more than 24 hr, or have been frozen and thawed for examination, is often inferior and difficult to identify. Trematodes and cestodes from frozen or formalinized hosts are often badly contracted and have lost structures crucial to identification like oral spines on trematodes or the hooks on the scolex of tapeworms. However, the reality is that most material available from vertebrate hosts these days is frozen or preserved.

Databases containing DNA sequence information for helminths is quickly growing and thus taxonomic identification is already possible for many species. Therefore, helminths should be preserved for potential DNA analyses as much as possible. However, DNA of good quality is not possible if specimens are fixed in formalin. Methods outlined here for collecting and killing helminths will yield material that is suitable for DNA extraction and genetic analyses.

Below, we describe detailed methods on how to necropsy vertebrates (amphibians, reptiles, birds and mammals; monogenean trematodes from fish are not included) for the collection of helminths, followed by procedures on how to preserve and process them for taxonomic identification.

Protocol

Animals used in the following experiments were found dead as road kills.

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1. Animal Necropsy and Screening of Major Organs for Helminth Collection

1. Position the animal on a flat surface and examine all external openings including ears, oral cavity and eyes with a magnifying glass for the presence of nematodes (round) and trematodes (flat and not-segmented) (see **Figure 1**).
2. If working with birds, wash with tap water to dampen the feathers prior to dissecting.
3. Using a sharp blade or knife and with the aid of a pair of dissecting forceps, make an incision over the abdominal cavity being careful not to rupture any organs. For turtles with a hard carapace, use a handheld saw to cut through the bone at the bridge area between the pectoral and abdominal scutes.
4. Open the thoracic cavity as above; for large animals, bone cutters or a small handheld-saw might be needed to cut through the ribs.
5. If working with birds, examine air sacs *in situ* prior to removing any organs. Use a magnifying glass if needed.
6. Examine both cavities for filarial roundworms and large trematodes.
7. Prior to removing the gastrointestinal tract from the abdominal cavity, tie a thread close to the beginning of the esophagus and another one at the end of the large intestine (rectum).
8. Separate the following thoracic organs into Petri dishes (or glass trays if organs are too large) for examination under a stereo microscope (10-30X): heart and major blood vessels, trachea, and lungs.
9. Remove liver, gall bladder and duct, pancreas, spleen, kidneys and urinary bladder and examine as described in step 1.8.
10. Remove the complete digestive system and place each section in glass dishes/trays.
11. After organs have been removed, wash the body cavity with a hose or squirt bottle filled with 0.9% saline into a 106 µm mesh sieve and examine the sieve contents under a stereo microscope for the presence of filarial worms or blood trematodes.
12. Open each digestive tract section with scissors, scrape the mucosa and examine carefully for the presence of large parasites (>1 cm). Use a magnifying glass if needed. The probosci of acanthocephalans are often deeply imbedded in the wall of the intestine and may have to be carefully teased from the tissue with forceps and/or small scissors.
13. Place each opened section under running water and wash all contents into a 106 µm sieve as described in step 1.11.
14. Open the heart and major blood vessels (pulmonary and aorta), trachea, and urinary and gall bladders and examine contents in the same way.
15. Slice and tease apart solid organs such as lungs, liver, kidneys, spleen, and pancreas using a sharp blade and forceps. Wash them under running water into a 106 µm sieve as described in step 1.11.
16. Record the types of parasites found, the number of each type, and the organ(s) in which they are found. Fixation and staining methods are listed below.
17. Label vials/jars by placing a small piece of a plain index card written with pencil (ink and ruled index cards will "run" in alcohol and stain specimens).

2. Preservation of Helminths

1. For helminths that need to be preserved for later genetic studies, place them directly into 80-90% ethanol (without glycerine). The ethanol should be made by diluting 95% ethanol, preferably reagent grade (**Table 1**). **Never allow these specimens to be exposed to formalin, as formalin degrades DNA to an unusable state.**
2. Place **live trematodes** on a glass microscope slide in a drop of saline, place a glass cover slip and pass the slide over a flame without boiling the saline.
    1. Drop the slide into a Petri dish containing AFA (alcohol-formalin-acetic acid, see **Table 1**) and let the cover slip float off.
    2. Fix in AFA for one to several days, rinse with tap water, and transfer to 5 ml glass vials (or larger glass vial/jar depending on size and amount of parasites) filled with 70% ethanol for long-term storage.
3. Fix **dead trematodes** in AFA or 10% buffered formalin for 48 hr and then transfer to vial/jar filled with 70% ethanol for long-term storage.
4. Relax **live cestodes** in a Petri dish by pouring near-boiling water over them and then transfer to vial/jar filled with 70% ethanol for long-term storage.
5. Relax **live acanthocephalans** by placing in a Petri dish for one to several hours in tap water in a refrigerator (~4 °C) until the proboscis is fully everted (this may take up to 24 hr). Transfer to vial/jar filled with 70% ethanol or AFA for long-term storage.
6. Treat **dead cestodes and acanthocephalans** the same as dead trematodes (see above).
7. Kill **live small (<5 mm, or fragile nematodes** (*e.g.* trichostrongylids, capillarids) in hot (near-boiling) 70% ethanol and preserve in vial/jar filled with 70% ethanol with 5% glycerine (**Table 1**) added to preserve the worms in case of evaporation.
8. **Kill (and clear) medium to large (>5 mm) nematodes** (*e.g.* ascarids, spirurids, oxyurids, filarids) by placing in a Petri dish with cold glacial acetic acid (**Table 1**) and after 15 min transfer to vial/jar filled with 70% ethanol and 5% glycerine. The acetic acid can be used repeatedly in this way.
9. Place **dead nematodes** directly in vial/jar filled with 70% ethanol and 5% glycerine.

3. Taxonomic Identification of Helminths

1. Prepare Harris' hematoxylin by dissolving hematoxylin in alcohol and by dissolving ammonium aluminum sulfate in water with heat. Mix both solutions and bring to a boil.
    1. Add sodium iodate and boil for 2-3 min. Filter (541 filter paper) solution after it is back to room temperature.
2. Overstain trematodes, cestodes and acanthocephalans by leaving them in diluted (1:10) Harris' hematoxylin or Semichon's carmine overnight (see **Table 1**).
    1. De-stain using 70% acid ethanol until organs are visible (can take between a few seconds to over an hour and should be closely observed).
    2. Halt de-staining by transferring specimens to 70% basic ethanol for at least 30 min. Next, dehydrate them in a series of graduated ethanols (80%, 95%, 100%) and clear them in xylene or methyl salicylate.

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3. Mount specimens on a microscope slide after adding a drop of Canada balsam and a cover slip. Let dry overnight.

3. Clear small to medium nematodes and acanthocephalans for 15-30 min in temporary mounts of lactophenol on a microscope slide with a cover slip.
    1. Use 80% phenol for up to 60 min for large nematodes (ascarids and spirurids) and acanthocephalans. Examine under a light microscope (40-400X) for evaluation of smaller structures (male spicules and hooks).

4. Use the following approaches for examination of special structures.
    1. For cestodes, cut off the scolex and place on a microscope slide in lactophenol and "squash" with a cover slip for measuring and counting rostellar hooks (see **Figure 1B**).
    2. For nematodes, cut off the anterior end and mount *en face* on a microscope slide in a few drops of lactophenol (without coverslip) or in glycerine jelly for a permanent mount (with cover slip) and observe mouthparts under a light microscope (35-400X) (see **Figure 1D**).
    3. Cut off the tails of large nematodes and mount on a microscope slide in lactophenol with a cover slip to observe the ventral papillae and spicule morphology of males under a light microscope (see **Figure 1D**).

5. Identify parasites to species using a combination of primary literature and standard taxonomic references such as:
    1. Trematodes: Yamaguti (1958, 1971)[3,4] and Gibson *et al.*[5-7]
    2. Acanthocephalans: Yamaguti[8]
    3. Cestodes: Yamaguti[9], Schmidt[10], and Khalil *et al.*[11]
    4. Nematodes: Yamaguti[12] and Anderson *et al.*[13]

4. Depository of Helminths in U.S. National Parasite Collection

1. Deposit representative helminth specimens at the U.S. National Parasite Collection (USNPC). This is mandatory for most parasite journals. After submission, each helminth species is assigned an accession number for inclusion in manuscripts.
2. Include the following information when submitting specimens: 1) Scientific name of parasite; 2) host information (species, longitude/latitude coordinates of geographic location); 3) organ found in host; 4) name of who collected the specimens and date of collection; 5) type of fixative, stain, or clearing/mounting media used; 6) location of other collections (museum and accession numbers) if additional specimens are submitted elsewhere; and 7) information on publication and journal. These data should also be sent together with the specimens and under separate cover.
3. Package specimens in strong containers with shockproof absorbent material. Tape/Parafilm screw caps on glass vials and place each vial in a separate plastic bag. Wrap slides in paper and place in a slide box with shock-absorbing material between them.
4. Ship specimens and separate letter with information about specimens to: U.S. National Parasite Collection, USDA, ARS, ANRI, Bldg. 1180, BARC-East, 10300 Baltimore Avenue, Beltsville, MD 20705-2350.

Representative Results

Using the methods outlined above, a survey of the helminths of the masked shrew, *Sorex cinereus*, was conducted in Missoula County, Montana between 2007-2011. A total of 56 shrews were collected from pitfall traps and examined within 2 hr after death. Overall prevalence of infection was 96%; only 2 shrews were free of parasites. Fifteen species of helminths were identified, including 9 species of cestodes and 6 species of nematodes. One species of the cestode genus *Staphylocystoides* was previously undescribed. Organs found infected included the small intestine (9 cestodes, 3 nematodes), stomach (1 nematode), lungs (1 nematode), and urinary bladder (1 nematode). Total intensities of infection ranged from 7-234 worms per infected host. Species richness (the number of helminth species per infected host) ranged from 1-8 with a mean of 4.1 (**Figure 2**).

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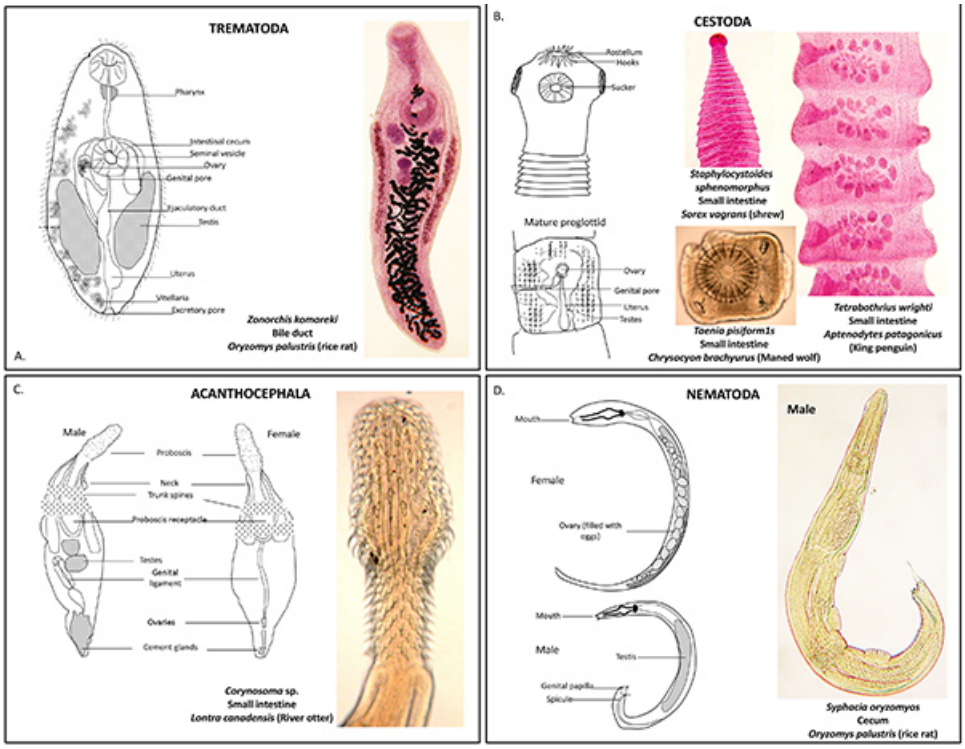

Figure 1. Representative drawings and photographs of helminth groups showing major anatomical features for each Phylu.: **A**) Trematoda; **B**) Cestoda; **C**) Acanthocephala; and **D**) Nematoda. For the photographs, information on the scientific name, organ and host are included. Trematodes and cestodes are hermaphrodites. Drawings are not necessarily on the same scale.Click here to view larger image.

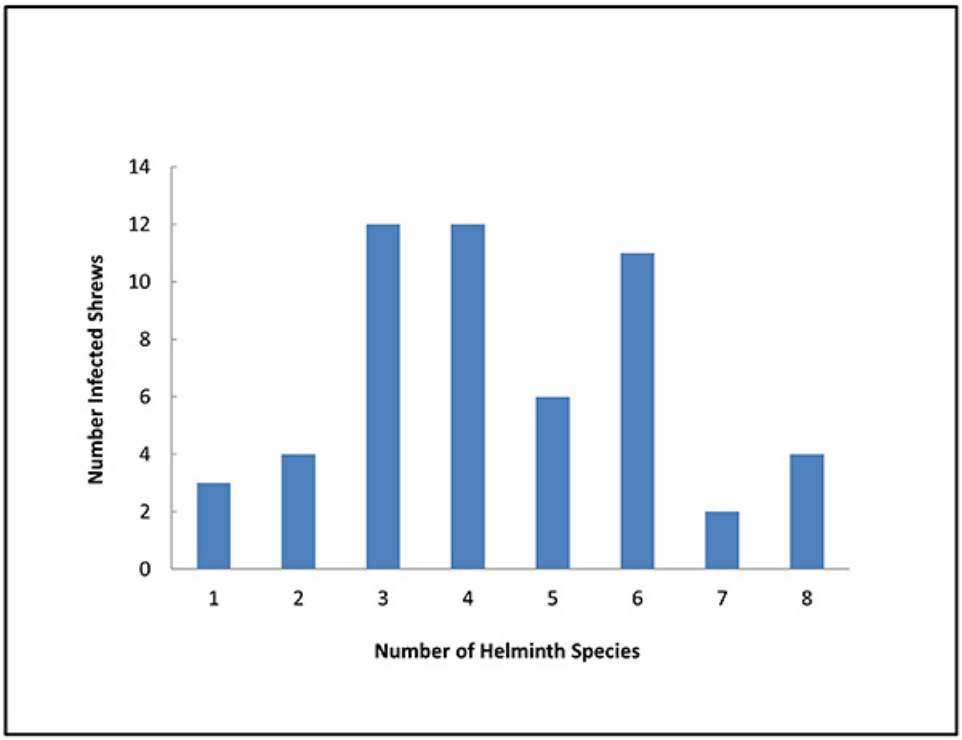

Figure 2. Summary of preliminary helminth data from masked shrews, *Sorex cinereus* collected from Missoula County, Montana between 2007-2011. Click here to view larger image.

Discussion

It is extremely difficult to identify helminth parasites, even to genus, based on poor material. Cestodes and trematodes, in particular, tend to die and deteriorate fairly rapidly after the death of the host. The taxonomy of cestodes depends greatly on the number, size, and shape of rostellar hooks, which are often lost in frozen material. The same applies to certain trematodes with spines around the oral sucker such as echinostomes and heterophyids, which are also frequently lost. Because of their thick cuticles, nematodes and acanthocephalans are a little hardier, but still may be contracted or have the proboscis retracted. Therefore, the importance of obtaining the freshest possible material cannot be overemphasized. Optimally, live hosts should be captured, euthanized and examined immediately. Failing that, hosts should be examined within 3-4 hr after death, or frozen as quickly as possible. Removing intestinal tracts immediately after collecting hosts and flash-freezing them with liquid nitrogen has yielded excellent material. If even a few hosts can be examined freshly and the helminths properly identified, this may allow the sorting of species collected from subsequent frozen material. Helminths can move within a host after death, and this needs to be taken into account when describing location of parasites during necropsy.

The use of a sieve to screen intestinal contents and to wash blood from other organs is a significant improvement over earlier methods of necropsy. It greatly increases the chances of finding the smaller helminths and improves quantitative analysis. Killing cestodes and trematodes in hot water yields specimens that are better for staining than those previously obtained by relaxing in cold tap water in a refrigerator.

The critical step in the staining of helminths is in the destaining process in acid ethanol and this is directly related to the type of parasite and its size. If the specimen is not destained enough, organs may not be able to be differentiated and if it is destained too much, organs may not even be visible. There is a certain art to this process and the only way to learn it is through trial and error. Specimens need to be monitored carefully under the dissecting scope while destaining. When multiple specimens of a species are available to be stained, a few may be removed from the acid ethanol at intervals to achieve a graduated series and the best specimens selected after clearing. If specimens appear to be destained too much, the whole process may be repeated by placing them back in the stain overnight.

The future of helminth taxonomy will be heavily influenced by DNA studies. The DNA database for helminth species is rapidly expanding and allows specimens collected today to be identified in the future. Therefore, it is important to preserve (in formalin-free fixative) a subset of specimens in ethanol for DNA extraction. The methods outlined here for collecting and killing helminths will yield material that is suitable for DNA extraction and genetic analyses.

Disclosures

We have nothing to disclose

Acknowledgements

The authors would like to thank Dr. Joe N. Caudell, Disease Biologist for the Indiana USDA APHIS Wildlife Services Program at Purdue University, for providing specimens used for the collection of helminths for the production of this video. Jennifer Serafin prepared all of the chemical solutions.

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