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A METHOD FOR THE COLLECTION AND PREPARATION OF DICYEMID MESOZOANS

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ABSTRACT: A simplified method designed to collect and prepare dicyemid mesozoans from cephalopod renal appendages, or «kidneys», and composed of 2 steps (preparation and staining of smears) is described. Usual problems with dicyemid slides and techniques for preparation of stained permanent mounts of mesozoan smears are also provided.

KEY WORDS: Dicyemid mesozoans, cephalopods, renal appendages, collection and staining.

INTRODUCTION

The dicyemid mesozoans are a relatively small and puzzling group of minute, elongate worm-like parasites with a simple yet multicellular structure and without definite affinities in the animal kingdom (HOCHBERG, 1990). HORI & OSAWA (1987), studying the origin and evolutionary relationships of the major groups of organisms as deduced from 5S ribosomal RNA sequences, included the mesozoans as a separate group between protozoan Ciliata and metazoan Platyhelminthes.

The dicyemids are the most common and characteristic parasites of the excretory organs (fluid-filled renal sacs, renal coelom or «kidneys») of cephalopod molluses. In decapod cephalopods (squids, sepiolids, and teuthoids), they have also been found in the reno-pancreatic coelom and occasionally in the pericardium (HOCHBERG, 1983).

A total of about 50 species of cephalopods, representing 18 genera, are currently known to be hosts of dicyemids (HOCHBERG, 1982). The parasites occur mostly in cuttlefish, sepiolids, and octopods and less frequently in loliginid squids and have been reported from temperate, polar, and subtropical waters in many parts of the world (SHORT, 1991).

Morphogenetic studies of dicyemid mesozoans may provide clues to the development of many-celled organisms (LAPAN & MOROWITZ, 1972, 1975): the differentiation of cells and the development of the embryo with an organism that represents the simplest known level of multicellular organization. They may also be useful in the elucidation of complex taxonomic problems in the cephalopod host (PASCUAL & HOCHBERG, 1996). In what is now a classic study, PICKFORD & MC CONNAUGHEY (1949) distinguished a sibling species complex of 2-spotted octopuses off California on the basis of the dicyemid parasites.

Rather than present here the different interpretations on general taxonomy, systematics, and the extremely complex morphogenesis and life cycles, it has seemed expedient to take up a simple method for collecting and preparing dicyemids for light microscopy studies. The reliability of this method was routinely assessed during a 5-year Systematics Ecology Programme of cephalopod parasites in waters off Spain.

DESCRIPTION OF THE METHOD

PREPARATION OF DICYEMID MESOZOAN SMEARS

On shipboard or in the field

1) Kill cephalopod (octopus, cuttlefish, sepiolid or squid). The cephalopod host, if active, can be anesthetized by addition of 70% ethanol to sea water (alternatively $MgCl_2$, $MgSO_4$). It is then laid on its back and a midventral cut of the mantle is made from its anterior edge posteriorly to expose the conspicuous, fluid-filled renal sacs, which lie at the surface of the visceral mass. The renal sacs (brownish-coloured spongy organs) are semitransparent, and within them lie the renal appendages (see Fig. 1), which are outpocketings of the venae cavae. Vermiform dicyemids adhere to the renal appendages, often with their calottes embedded in depressions in the renal epithelium. Smaller vermiform individuals occur within the folds and crevices of the appendages, and a few dicyemids may also occur free in the urine.

2) Place venous appendages of kidney in small amount of urine from host or in sea water (if sufficient urine is not obtained); gently tease apart renal appendages into small pieces with needle-nose forceps and examine with dissecting microscope; edges of infected kidney appear hairy under low magnification. «Hairs» are mesozoans.

3) Prepare smears on clean coverslips (1 or 1,5 thick type; 22 × 22 mm square) by brushing a piece of kidney

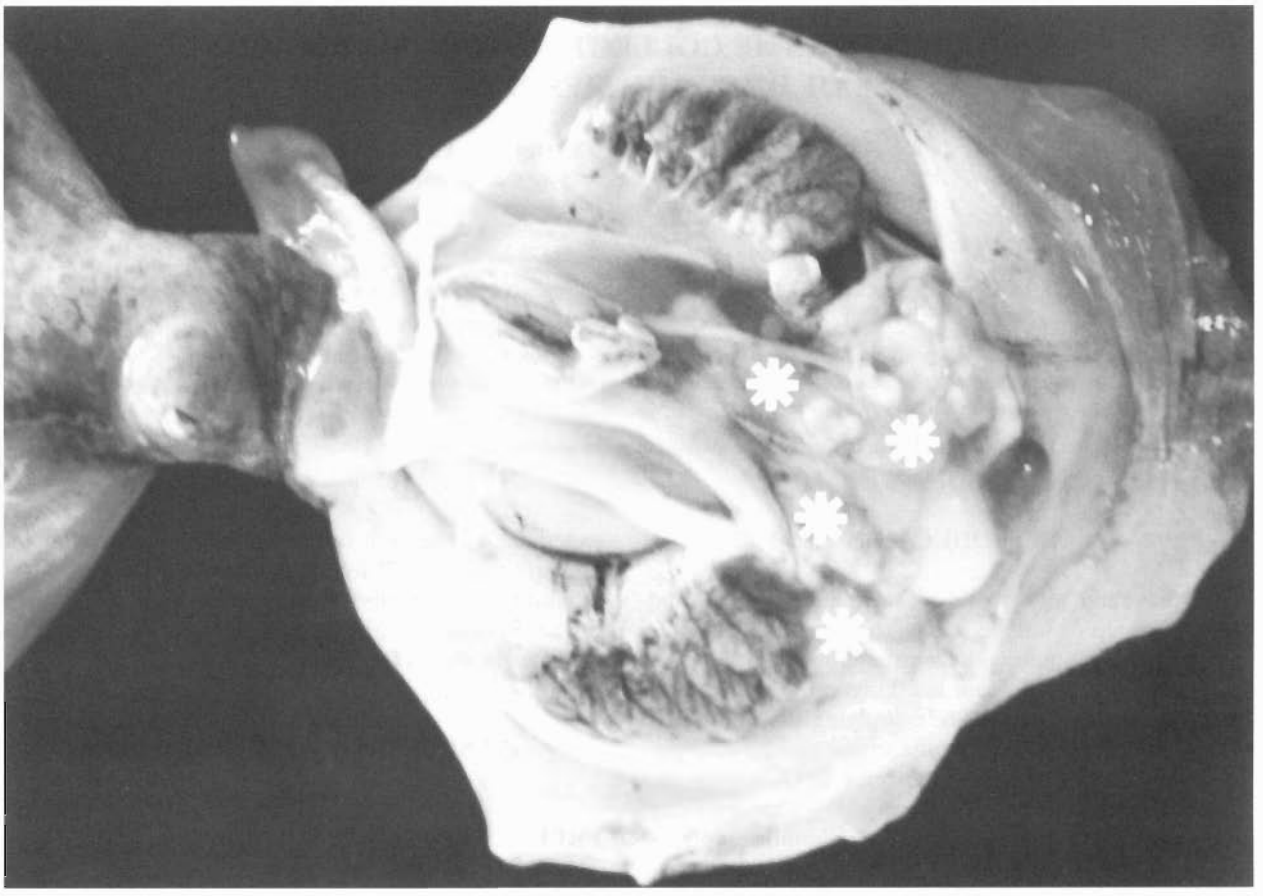

Fig. 1.– *Octopus vulgaris*, ventral view. Dissected to show renal appendages (asterisks) within semitransparent renal sacs.

gently in one direction over coverslip. Each small piece of kidney from an infected host can be used to make smears on one coverslip. Save the pieces of kidney after each piece is used. Make sure the parasites in the smears are enrobed with a coating of mucous. Be careful not to allow either the kidneys or smears to dry. Prepare at least 20 coverslip smears from each host. Drop coverslips, smear side down, in Petri dish of fixative. Pencil the cephalopod name and host number on label paper, the same size as the coverslip, and place in each Petri dish at the time of fixation.

4) When all the smears have been prepared, place remaining pieces of kidney and urine in 1-oz screw-cap vial; add equal amounts of 5% formalin and sea water (6%). Close vial and shake gently to dislodge mesozoans. Add label with host name and number; secure and tape lid.

5) Fix cephalopod host in 5-10% formalin. Enclose a label with host number, host name, size (mantle length and/or weight), relative maturity (paralarvae, juvenile, immature, submature, mature), age (when possible through examination of growth increments in the statolith), locality, date collected, collector and date autopsied.

6) Prepare a host data sheet for each specimen autopsied and examined.

For cytological preparation

7) Drop coverslips, smear side down, in Petri dish of fixative. Pencil the cephalopod name and host number on label paper, the same size as the coverslip, and place in each Petri dish at the time of fixation.

Sanfelice's Fixative (mix immediately before using)

Chromic acid 1%	16 pts.
Formalin, concentrated (40%)	8 pts.
Acetic Acid, glacial	1 pt.

Material can be left in Sanfelice's more or less indefinitely. See step 8.

Bouin's Fixative

Picric Acid, saturated aqueous	75 pts.
Formalin, concentrated	25 pts.
Acetic Acid, glacial	5 pts.

Allow coverslips to float in Bouin's for 10 to 24 hours; transfer material to 70% ethyl alcohol and proceed as directed in step 8.

Sanfelice Fixative	**Bouin's Fixative**
wash in dist. H_2O - several changes	wash in dist. H_2O + Li_2CO_3 (until white) wash in dist. H_2O - several changes
30% ETOH (ethyl alcohol) 50% ETOH store in 70% ETOH* 50% ETOH 30% ETOH wash in dist. H_2O	30% ETOH 50% ETOH store in 70% ETOH* 50% ETOH 30% ETOH wash in dist. H2O
Mordant: 2.5% fresh Iron Alum (1hr.) wash in tap H_2O (several changes) wash in dist. H_2O (3-5') Stain: Haematoxylin (0.5% aqueous) (1.5 hrs.: 10% stain in 100% ETOH - 5 pts; dist. H_2O - 95 cc; FILTER after mixing) wash in tap H_2O (several changes)	**Stain**: Ehrlich's Acid Haematoxylin (8 hrs.) diluted: stain stock - 1 pt dist. H_2O - 20 pts (FILTER after mixing) wash in tap H_2O - several changes wash in dist. H_2O 30% ETOH (1') 50% ETOH (1')
Destain - sat. aqueous Picric Acid (till destained sufficiently) wash in tap H_2O - several changes wash in tap H_2O + 2 ml Li_2CO_3 wash in tap or dist. H_2O wash in dist. H_2O 30% ETOH 50% ETOH 60% ETOH 70% ETOH 85% ETOH	**Destain** - 70% ETOH + acid (dilute HCl. 0,1%) 85% ETOH + K Acetate (1')
95% ETOH (1') 95% ETOH + Esoin (10') 95% ETOH - wash briefly (1') 100% ETOH (1') 100% ETOH - (1') Xylol (1') Xylol (1') Mount: Canada balsam, DPX, Damar, Permount or Piccolyte	

Table 1.– Technique for preparation of stained permanent mounts of mesozoan smears. * = can be stored indefinitely in 70% ETOH.

8) When convenient, transfer coverslips to small, wide-mouth, screw-cap jars, smear side down (one jar with coverslips from each cephalopod host). Stack the coverslips, smear side down, and separate each coverslip with a square piece of gauze (cheese cloth) to prevent their sliding over each other or sticking together. Include a label with host name and number on top in each jar. Seal jar tightly and tape lid. Smears can be stored or shipped in these jars. Smears fixed in Sanfelice's can be stored and shipped in Sanfelice's; smears fixed in Bouin's can be stored and shipped in 70% ethyl alcohol.

In the laboratory

9.) Smears should be stained with haematoxylin, e.g., Ehrlich's acid haematoxylin or Heidenhain's iron haematoxylin (or some other suitable stain), and, if desired, by counterstaining with eosin or acid fuchsin (1%; 2 hours). Mount on microslides using Canada balsam, DPX, Permount or Piccolyte. Care should be taken not to let the coverslip smears become dry at any time during the process from fixative to mounting medium. Mark host number on edge of final prepared microslide with a diamond stylet.

10) Label slides appropriately, e.g. as indicated in Fig. 2.

0405-1 HOST: OCTOPUS VULGARIS RIA DE VIGO (NW SPAIN)	**SMEAR**	**DICYEMA TYPUS** TYPE MATERIAL BOUINS, EAH. **F-6.** PASCUAL.

Fig. 2. An example of correctly labelled microscope slide.

USUAL PROBLEMS ON DICYEMID SLIDES

Problems with smears: If the cephalopod hosts are obtained from commercial landings (i.e., dead), very few dicyemids are present on the slides, with lots of cellular debris. Either the kidneys are starting to decompose or the tissue was too wet when making the smears and many of the parasites can be washed off.

Air bubbles present under the coverslips: As a result the slide material is dried out in large areas. The bubbles usually developed after the coverslips are mounted on the slides. It appears that not enough mounting medium is used or with the medium one may have to ring the slide to prevent bubble from forming and connecting to the outside.

Not enough stain: This is either a problem of not leaving the coverslips in stain long enough or the smears having been destained too long. The destaining time is critical and the optimal time should be determined before all the good smears are processed. The destaining time seems to depend on the strength of the stain. Destaining should be monitored under a compound microscope under the 10x objective of an old microscope. The coverslips are smear-side up in a Petri dish of picric acid solution. Destaining is a matter of practice, and no hard and fast rules can be given.

Using a counter stain of eosin is also recommended. Also, do not use an old stain stock, i.e., stock that had aged and is not freshly made. Stain from freshly mixed stock often does not stain very well.

CONCLUSION

The simplified method here described provides large quantities of preparations of dicyemid mesozoans from cephalopod excretory appendages using an easy and inexpensive technique. Following this method the vermiform stages (worm-like forms including the stem nematogens, nematogens, rhombogens, and vermiform embryos), infusorigen and the infusoriform larvae preserved their morphological and tintorial characteristics in good condition for studies on morphology, morphogenesis, embryology and chromosome life cycles.

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