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An improved method for isolating camallanid (Nematoda) spicules for scanning electron microscopy

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Short Communication

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Abstract

In nematodes, the structure of male copulatory organs is a significant taxonomic distinguisher and includes the morphometry of the spicules. The description of these structures mainly relies on the study of whole mounts using light microscopy. In rare instances, protruding spicules have been described with scanning electron microscopy. Even fewer studies have described the ultrastructure of isolated spicules following their isolation. In the present study, two different methods of spicule isolation were performed on two parasitic camallanid nematodes, *Procamallanus (Procamallanus) pseudolaeviconchus* Moravec & van As, 2015 and *Paracamallanus cyathopharynx* (Baylis, 1923), from African sharptooth catfish to determine the practicality and efficiency of the methodologies. The first method involved using sharpened tungsten needles and microdissection of the spicule pouch to free the spicules, followed by soft tissue digestion if necessary. Alternatively, the spicules were isolated through mechanical release instead of dissection in a method developed in the current study. This involved freeing the spicules from surrounding soft tissue by placing live specimens between a coverslip and a glass slide in a drop of water and exerting pressure with small rotational movements. Both methods yielded favourable results, but Method 2 is recommended for future studies due to the many advantages.

Introduction

Generic and species differentiation of male nematodes generally requires information about the morphometry of copulatory structures such as the spicules. Features mostly included are the length and number of spicules (e.g. Moravec, 1974, 1975; Ivashkin *et al.*, 1977; Boomker, 1982), and later, detailed information about the spicule including their shape and tip characteristics (e.g. Scholz *et al.*, 2018; Moravec, 2019; Svitin *et al.*, 2019; Rindoria *et al.*, 2020). Most observations of these copulatory structures are of intact specimens using light microscopy (LM). However, this approach can be limiting due to the mounting, clearing or orientation of specimens, as well as other tissue/organs, obscuring the gross morphology of copulatory structures in the whole nematode (Rammah & Hirschmann, 1987). Gradinarov (2003) exposed male nematode specimens to heated 10% potassium hydroxide and studied cuticle remains and spicules using LM. In other instances, protruding spicules were serendipitously studied using scanning electron microscopy (SEM) (e.g. Moravec & Jirků, 2017; Moravec & Scholz, 2017; Rindoria *et al.*, 2020; Al-Bassel *et al.*, 2022) and these studies revealed additional detail. On rare occasions, spicules were isolated through dissection from the spicule pouch of specimens in glycerine or lactic acid, which were then studied with SEM (Eisenback, 1985; Rammah & Hirschmann, 1987). This process has not gained much traction, presumably due to the technical skill required. The present study isolated the unequal spicules, referred to as the left and right spicule, from the males of two parasitic camallanid nematode species from fish – *Procamallanus (Procamallanus) pseudolaeviconchus* Moravec & van As, 2015 and *Paracamallanus cyathopharynx* (Baylis, 1923) – using and optimizing available methodology.

Materials and methods

Parasitic nematodes were collected from *Clarias gariepinus* (Burchell, 1822) in the Crocodile River, North-West province, South Africa (Nofal *et al.*, 2022). Specimens were fixed in either steaming hot 70% ethanol, room temperature 70% ethanol, steaming hot triethanolamine formalin or steaming hot 10% neutral buffered formalin, while other specimens were processed live. Spicules were isolated using one of the following two methods.

Method 1

Following Austin *et al.* (2023), the method from Rammah & Hirschmann (1987) was adapted. Fixed specimens (50 male nematodes; 25 of each species) were transferred to concavity glass

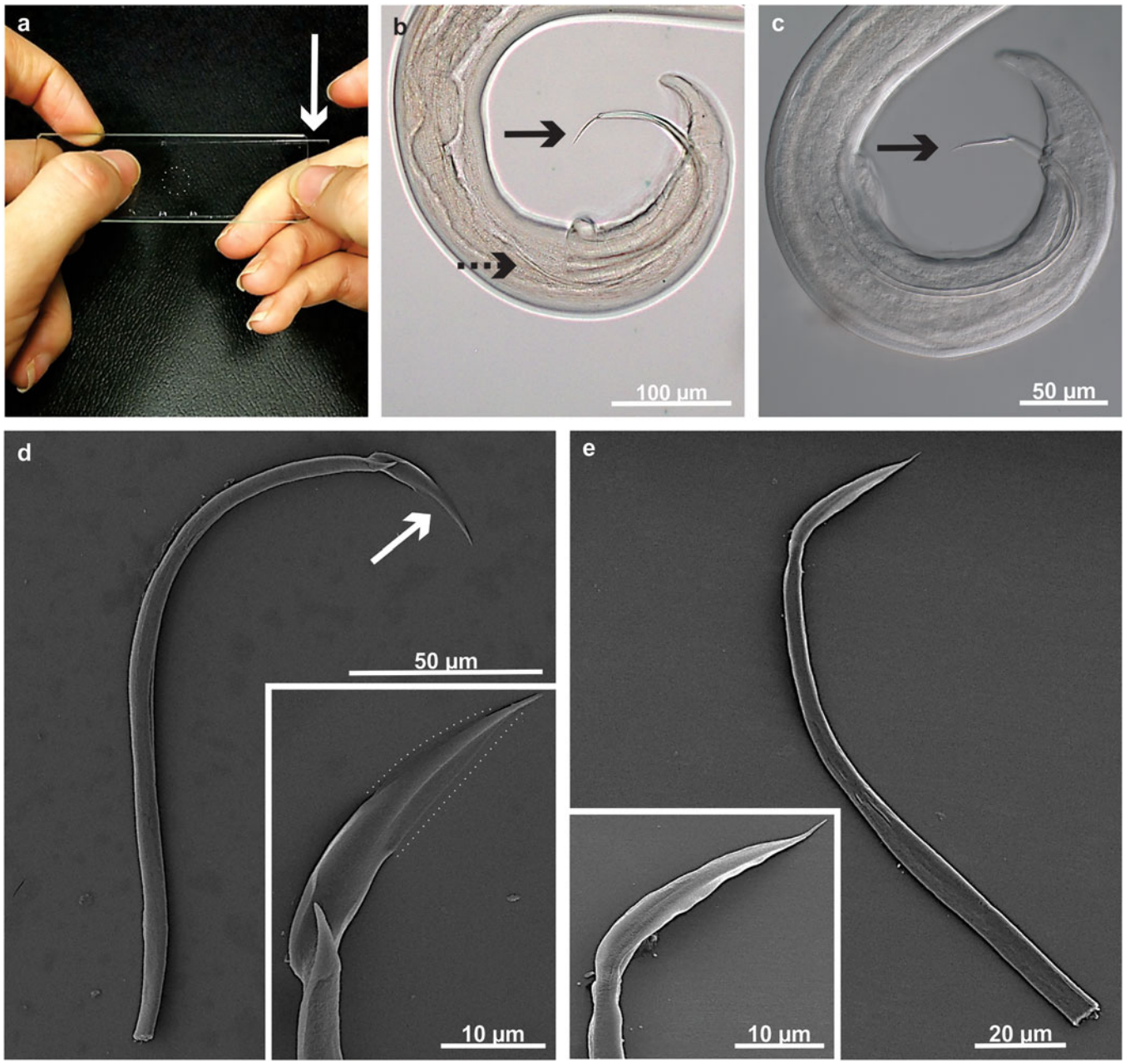

Fig. 1. (a) photograph of the coverslip–slide complex used in Method 2 – the arrow shows the placement of the overhanging coverslip and the dashed circle shows the position of the specimen; (b–g) light (LM) and scanning electron (SEM) micrographs of the tail region and isolated spicules of male *Paracamallanus cyathopharynx* (Baylis, 1923): (b) LM of tail region – solid arrow shows right (large) spicule and dashed arrow shows left (small) spicule; (c) LM of the tail region using differential interference contrast – arrow shows right (large) spicule; (d) SEM of isolated right (large) spicule – arrow shows velum adhering to the glass slide and inlay highlights adhered velum with dotted lines; and (e) SEM of the alternate surface of the ethanol-fixed isolated right (large) spicule – the inlay shows the unadhered velum.

slides and immersed in 40% lactic acid or lactophenol and left to clear. Using a dissection microscope (10×, 4×, 2× magnification), the posterior regions were separated from the rest of the body using two sharpened tungsten needles (Brady, 1965; Bowen, 2010), securing the nematode with one needle and cutting with the other. Both the left and right spicules were released by systematically micro-dissecting the spicule pouch using the needles to tear away obstructing tissue.

Any remaining soft tissue was removed through soft tissue digestion using an enzymatic tissue digestion solution following the camallanid nematode buccal capsule isolation protocol of Rindoria *et al.* (2020) using the proteinase-K based digestion buffer from an E.Z.N.A DNA extraction kit (Omega Bio-tek, Inc. Norcross, USA). After that, isolated spicules were rinsed with 2% formalin (Rammah & Hirschmann, 1987) to remove any remaining lactic acid and buffer. Isolated spicules were dried overnight in the concavity glass slides in a Sanplatec dry keeper desiccator cabinet (Sanplatec Corporation, Japan), sputter coated with gold using an Emscope SC500 sputter coater (Quoram Technologies, Newhaven, UK) and studied using a TESCAN Vega 3 LMH (Tescan, Brno, Czech Republic) scanning electron microscope at 8 kV.

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Method 2

Three alternative versions were attempted

a) Using live specimens, nematodes were transferred to a drop of Milli-Q water on a microscope glass slide and the posterior separated from the rest of the body using sharpened tungsten needles. The remaining nematode tissue was removed and stored in 96% ethanol for other applications. A 22 × 60 mm coverslip was placed onto the drop, containing the posterior section of the nematode (fig. 1a). The coverslip was placed in such a way that part of the coverslip was hanging over the short edge of the glass slide. The coverslip was then manually rotated in small circles, adding gentle pressure, if necessary, until both spicules were released from the tissue. Once isolated, the coverslip was removed, and 70% ethanol dropped over the spicules to fix the structures. The position of the spicules, on either the coverslip or glass slide, was marked with a permanent marker, stored in a desiccator overnight to dry, and studied using SEM as described above, except using microscope glass slides instead of concavity slides.

b) To limit the loss of spicules when removing the coverslip post-isolation, the entire coverslip–slide complex was stored in a desiccator overnight, after dripping sufficient 70% ethanol on the sides of the coverslip to replace the Milli-Q water. When dry, the coverslip was removed from the slide while observing with a dissection microscope and the isolated spicules studied using SEM as described above.

c) In the event that fresh material was not available, specimens in glycerol were also used. The glycerol was removed by rinsing the specimens with distilled water before processing, as described above.

If required, spicules could be reorientated using an eyelash hair attached to a toothpick. Repositioning was also possible after initial SEM study, including sputter coating with gold. This allowed for the study of the opposite surface of the spicules.

Results and discussion

Both methods for spicule isolation provided good results; however, each had its advantages and disadvantages. Firstly, Method 1 required the accurate identification of the location and extent of the spicules within the whole nematode and to clearly distinguish between the left spicule and the right spicule (fig 1b, c), which was more challenging for the left spicule due to the limited magnification of a dissection microscope. Secondly, Method 1 required advanced fine-motor skills to free the spicules from tissue, with improper manipulation resulting in damage, breakage and/or loss of the spicules, necessitating many specimens to obtain ideal results. This method is, therefore, deemed impractical where a limited number of specimens are available. Furthermore, the large number of specimens needed for Method 1 required persistent sharpening of the tungsten needles. Enzymatic digestion of soft tissue, post microdissection, caused unintentional loss of structural integrity of the spicules and incomplete rinsing of the digestion buffer left residual material, obscuring structures.

Method 2 appeared superior to Method 1 as it required less advanced fine-motor skills due to the elimination of microdissection. Due to the ease and convenience of Method 2, it was, therefore, also more time-efficient, which is important when large sample sizes are studied. However, there was an initial high rate of spicule loss when removing the coverslip, but this was addressed by keeping the coverslip–slide complex intact during fixation as per Method 2b. Due to the manual rotations, the right spicule broke in some cases, probably owing to its size and shape. These rotations also resulted in the alteration of the natural shape of spicules on occasion. Method 2 also allows for using a digestion buffer after the spicules have been isolated to remove the remaining tissue. The use of Method 2 for specimens in glycerol was satisfactory; however, not as effective as using fresh material.

Eisenback (1985) reported that spicules could withstand air-drying as they are not hydrated, and usually sclerotized, and will therefore maintain their natural integrity. However, both Eisenback (1985) and Rammah & Hirschmann (1987) immersed live specimens in lactic acid or glycerine during spicule isolation and rinsed them with 2% formalin before drying. In this study (Method 2), live specimens that had not been exposed to any chemical were used, and the isolated spicules were initially dried unfixed. This caused structures such as the velum to adhere to the slide or coverslip, distorting the structure (fig. 1d). Therefore, 70% ethanol was added to isolated spicules in Method 2 to prevent this (fig. 1e). Both Method 1 and Method 2 enabled re-orientation of spicules (using an eyelash hairbrush) if a study of the alternate surface of some spicules is required, even after initial SEM (fig. 1e). This enabled observation of the ventral barb on the right spicule of *P. cyathopharynx*, which is only visible from one side.

Using these methods, isolated spicules can furthermore be studied using LM to provide paratype and voucher specimens as an additional method to the study of whole mounts. In this case, isolated spicules could be mounted using mountants such as glycerol (sealed with nail varnish or similar substance to prevent drying) or in Canada balsam. Importantly, it is suggested that these methods not be used when only a few specimens are available, especially in the case of a potential new species, as the entire posterior end of the specimen is destroyed. Therefore, morphometry (including illustrations) of the posterior end could be done before performing any of these methods if required.

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Conflicts of interest. All three authors contributed equally to the manuscript and agreed to the publication of the paper.

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