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Tegumental ultrastructure of *Nephrostomum ramosum* Sonsino, 1895 (Trematoda: Echinostomatidae) from *Ardeola ibis ibis*

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During an investigation on intestinal flukes of cattle egret, *Ardeola ibis ibis* excysted metacercaria and adult worms belonging to family Echinostomatidae *Nephrostomum ramosum* were recovered. The tegumental surface of *N. ramosum* was described for the first time using scanning electron microscopy (SEM). SEM observations of excysted metacercaria and adult worms showed some basic differences, excysted metacercaria is conical shaped, ventrally curved, the oral sucker is not developed and the ventral sucker is located in the anterior third of the body, while adult worm has an elongated body with a distinct head collar at the central of which is located on the subterminal oral sucker. The oral and ventral suckers are located closely in the anterior fifth of the body. The total number of collar spines is 40 including five end group ones on each ventral corner. The tegument of the body is wrinkled with transverse grooves and is devoid of spines. The surface of whole body is lacking sensory papillae except the dorsal area of the cephalic region. The results reveal that the characteristic features of *N. ramosum*, including the number and shape of collar spines, the corrugated surface of the body, absence of tegumental spines and distribution of papillae differed from other echinostomes.

Key words: Trematode, echinostomatidae, *Ardeola ibis ibis*, tegument, scanning electron microscopy (SEM), *Nephrostomum ramosum*.

INTRODUCTION

Wild birds form a large branch of the animal kingdom and are very widely distributed. Many investigators in different parts of the world paid a great attention to studying the helminth parasites of wild birds (Nicoll, 1923; Ali, 1968; Mawson, 1977; Guta, 1983; Borgsteede, 1989; Tanveer and Chishti, 2001; Foronda et al., 2003; Kulisic et al., 2004). In Egypt, the helminth parasites of some wild birds have been studied by Azim (1935), El-Naffar et al. (1978), El-Sheikh and Hegazi (1984), Ahmed (1994) and Aboel Hadid and Lotfy (2007).

Nephrostomum ramosum (Family: Echinostomatidae) was recorded for the first time from cattle egret in Egypt by Azim (1934). Characteristics taxonomic of the family Echinostomatidae are the presence, number, size, shape and arrangement of collar spines. However, the information that is often contradictory is regarding the

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number and arrangement (Kostadinova, 2005). The number and arrangement of the collar spines found typically around the oral region of the adult as well as the larval stages, form the basis of the different species of echinostomes (Fried, 2001).

The tegument of echinostomes represents the major interface between the parasite and the host and is intimately involved in the relationship between the host and the parasite. The tegument can be implicated in different processes such as the attachment to the host intestinal mucosa, the interaction with the medium or the motility of the parasite. In this regard, several studies have been done to determine the relation between collar spine and parasite explusion (Kruse et al., 1992; Fujino et al., 1994), or the differences in tegumental spines development in relation to the host species (Sotillo et al., 2010). Kruse et al. (1992) and Fujino et al. (1994) reported that retraction of collar spines might play an important role in the explusion of echinostomes from the hosts.

The present study gives the first description of the tegumental ultrastructure of both excysted metacercaria and adult worms of genus *Nephrostomum* (Family: Echinostomatidae), that improve the knowledge of the topography of the tegumental surface as taxonomic characteristics of *N. ramosum*, and comparing it with other related genus.

MATERIALS AND METHODS

Ardeola ibis ibis were collected from Sharkia Governorate, Egypt, and were brought alive to the laboratory. Then the birds were sacrificed and dissected. One excysted metacercaria and ten adult worms of *N. ramosum* were collected alive from the small intestine of eleven *A. ibis ibis* (sin. *Bubulcus ibis*) (Aves, Pelecaniformes) For light microscopy, four specimens of *N. ramosum* were compressed, fixed in 70% ethanol, dehydrated in an crescent ethanol series, stained with aceto-carmine (Pearse 1972), cleared with clove oil, mounted in Canada balsam, covered with cover glass and left to dry in an oven at 40°C. The permanent slides of adult worms were examined and photomicrographs were taken. For scanning electron microscopy, one excysted metacercaria, five adult worms of *N. ramosum* were rinsed several times in saline solution, fixed in cold (4°C) 2.5% glutaradehyde (in 0.1 M sodium cacodylate buffer) at pH 7.2 for 3 h, rinsed repeatedly in 0.1 M sodium cacodylate buffer at pH 7.2 and post fixed in cold (4°C) 1% osmium tetroxide in the same buffer for 1 h, dehydrated in ascending grades of ethanol and critical-point-dried using liquid CO_2, dried specimens were coated with gold and examined with a Jeol JSM-5200 scanning electron microscope.

RESULTS

Description

Light microscopy observations revealed that, adult *N. ramosum* is an elongated fluke, with smooth cuticle

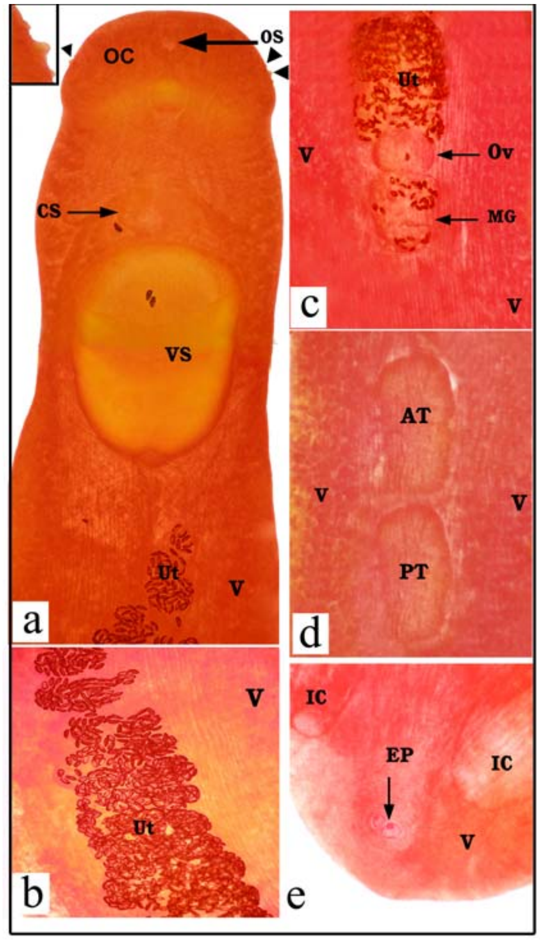

Figure 1. Light photomicrographs of adult *Nephrostomum ramosum*. **a)** Anterior part of the body showing the oral collar (OC), oral sucker (OS), cirrus sac (CS), ventral sucker (VS), uterus (ut) and vitellaria (V). Insert showing collar spines. Scale bar = 400 µm. **b)** First third of the body, showing uterus (ut) crowded with eggs and vitellaria (v). Scale bar = 400 µm. **c)** Middle part of the body, showing ovary (Ov), uterus (ut), Mehlis gland (MG) and vitellaria (V).Scale bar = 400 µm. **d)** Posterior-half of the body, showing the anterior testes (AT), posterior testes (PT) and vitellaria (V). Scale bar = 400 µm. **e)** The posterior extremity of the body, showing the two intestinal caecum (IC), excretory pore (EP) and vitellaria (V). Scale bar = 400 µm.

without spines. Oral collar is located terminally, well developed, kidney-shaped, bearing collar spines in a single row. Oral sucker is subterminal and rounded, ventral sucker is funnel-shaped, larger than oral sucker and located in the anterior quarter of the body. Cirrus sac is well developed, elongated and over lapped with the anterior margin of ventral sucker (Figure 1a). Uterus is coiled, filled with numerous eggs and occupying the distance between ovary and ventral sucker (Figure 1a, b and c).

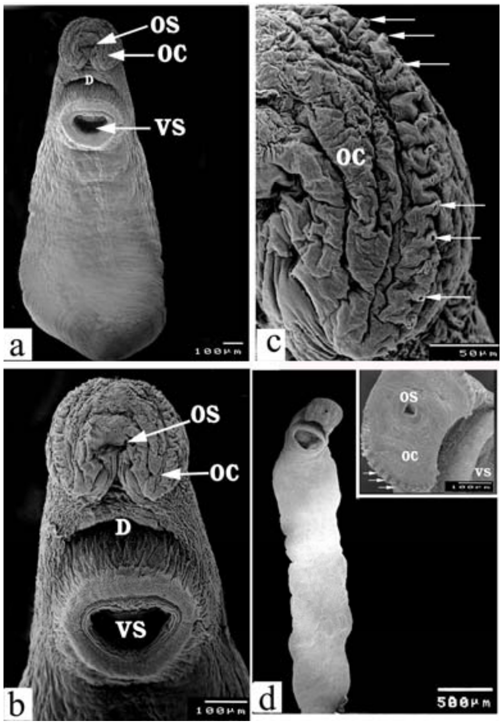

Figure 2. SEM of *Nephrostomum ramosum*. **a)** Ventral view of excysted metacercaria, showing oral collar (OC), oral sucker (OS) and ventral sucker (VS). Scale bar = 100 μm. **b)** Ventral view of anterior part of excysted metacercaria showing oral sucker (Os) depression (D), located between oral sucker (OS) and ventral sucker (VS). Scale bar = 100 μm. **c)** Enlarged view of oral collar of excysted metacercaria, showing surface of of oral collar (OC) and spinal pocketes (arrows). Scale bar = 50 μm. **d)** Ventral view of adult worm. Insert, showing enlarged view of oral collar (OC), with oral sucker (OS), ventral sucker (VS), depression (D) and spinal pocketes (arrows) Scale bar = 100 μm.

Ovary is spherical anterior to Mehlis' gland, on the median line of the body and Mehlis' gland larger than ovary (Figure 1c). Vitellaria extend laterally from lower level of ventral sucker up to posterior end of the body (Figure 1a - e). Testes are elongate, in tandem, postovarian, nearly equal in size and located behind the middle of the body (Figure 1d). Excretory pore is terminal at the posterior end of the body and intestinal ceca simple, smooth extending up to the posterior end (Figure 1e).

SEM of excysted metacercaria showed that it is conical shaped, ventrally curved and the ventral sucker is located at about the anterior third of the body (Figure 2a). The lip of oral sucker is not developed, whereas the lip of ventral sucker is prominent and thick, the outer rim of the ventral sucker is smooth, while the inner rim is highly folded, the area between the two suckers has a corrugated surface (Figure 2b). SEM of the excysted metacercaria showed the characteristic echinostomatid peristomic collar, but

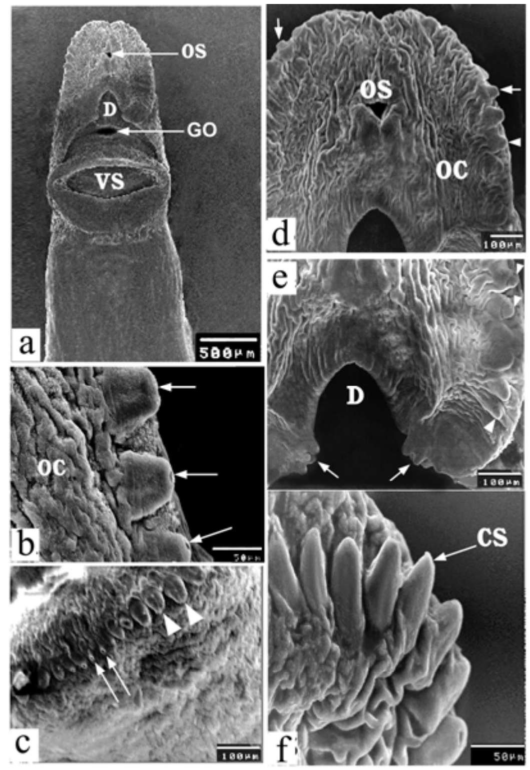

Figure 3. SEM of *Nephrostomum ramosum.* **a)** Ventral view of anterior part of adult worm, showing oral sucker (OS), funnel shaped ventral sucker (VS) and genital opening (GO) situated in depression (D) between oral and ventral suckers. Scale bar = 500 μm. **b)** High magnification of oral collar showing spinal pocketes (arrows). Scale bar = 50 μm. **c)** Lateral view of oral collar, showing partially protruded collar spines (arrow heads) and spinal pocketes (arrows). Scale bar = 100 μm. **d)** Ventral view of oral collar (OC), showing oral sucker (OS), partially protruded collar spines (arrow head) and spinal pocketes (arrows). Scale bar = 100 μm. **e)** Ventero-lateral view of oral collar, showing left and right corner spines (arrows). Note partially protruded spines (arrow heads). Scale bar = 100 μm. **f)** High magnification of cephalic collar showing tongue-shaped collar spines (Cs). Scale bar = 50 μm.

the collar spines are retracted into spinal pockets and could not be seen (Figure 2b and c). A depressed area between the oral collar and the ventral sucker is noted (Fig.2a,b).The tegument of whole body is mainly devoid of tegumentary spines (Fig.2a,b), the oral collar and the lips of both suckers are devoid of tegumentary papillae (Fig.2b,c) where the tegument of the oral collar has deep wavy wrinkles (Figure 2c).

SEM of adult worms showed it is elongated and its oral and ventral suckers are located closely in the anterior fifth of the body (Figure 2d). The lips of both suckers are well developed, prominent and thick, with smooth outer and inner rims in the ventral sucker, where the surface of the

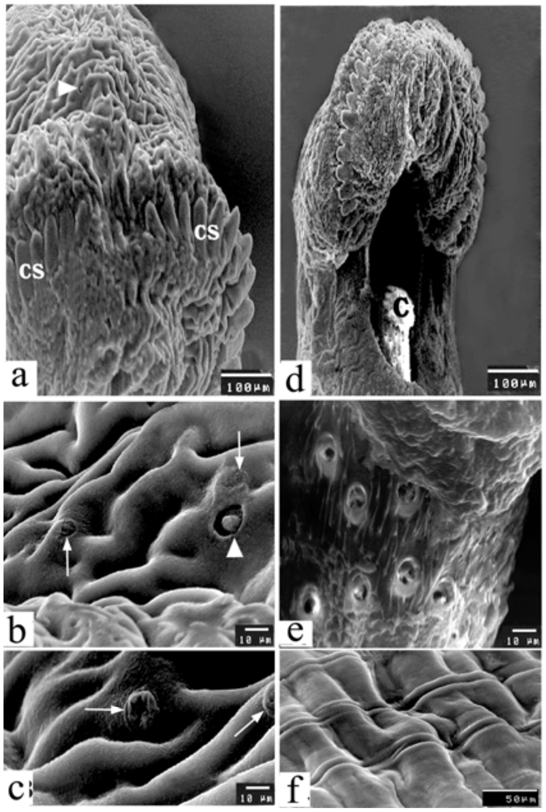

Figure 4. SEM of *Nephrostomum ramosum*. **a)** Tegument of dorsal surface, posterior to collar spines (CS), showing corrugated surface with sensory papillae (arrow head). Scale bar = 100 µm. **b, c)** Tegument of dorsal surface of cephalic region with sensory papillae. Button-shaped papillae (arrows) and styloconic-like papillae (arrow head) were seen. Scale bar = 10 µm. **d)** Ventero-lateral view of anterior part, showing protruded cirrus (C). Scale bar = 100 µm. **e)** Larger view of cirrus showing styloconic-like papillae. Scale bar = 10 µm. **f)** High magnification of body surface showing wrinkled tegument with transverse grooves. Scale bar = 50 µm.

area between the two suckers is smooth (Figure 3a). A depression area between the oral collar and the ventral sucker is noted (Figure 3a). The oral collar and the lips of both suckers are devoid of tegumentry papillae while the tegument of the oral collar has deep wavy wrinkles (Figure 3b and d).

SEM of adults showed some oral collar spines are retractable into spinal pockets and other are partially extended (Figure 3b,c and d), the collar spines appear as a tongue-shaped which are rounded at their tips and covered by the tegument layer (Figure 3e and f). The oral collar is bearing 40 collar spines and the collar spines are varied in the arrangement around the collar, that is., there are two groups of five corner spines arranged on each left and right ventro lateral lip of the oral collar (Figure 3e) and two groups of fifteen lateral spines are arranged in a single row on each left and right lateral surface of the oral collar (Figure 4d).

SEM of the adult showed that the tegument of whole body is devoid of sensory papillae except the dorsal surface of the cephalic region that possessed two type of papillae, button-shaped papillae and styloconic-like papilla, also the tegument of this region is highly corrugated (Figure 4a, b and c) The cirrus is protruded from genital opening (Figure 4d) and showed abundance of tegumentary styloconic-like papillae (Figure 4e). The tegument of the body is smooth, without spines and wrinkled with transverse grooves (Figure 4f).

DISCUSSION

It has been verified that *N. ramosum* is distributed indigenously among the cattle egret in Egypt (El-naffar and Khalifa, 1975; Hegazi, 1978; Ahmed, 1994; Aboel Hadid and Lofty, 2007). *N. ramosum* was reported from cattle egret on other countries (Nicoll, 1923; Rajvanshi and Gupta, 1983; Whittaker et al., 1971; Stuart et al., 1972).

In our study, the specimens of *N. ramosum* have 40 collar spines, this differs from that given by El-Naffar and Khalifa (1975), El-Naffar et al. (1978), Hegazi (1978) and Ahmed (1994) where they recorded 46 collar spines. The funnel shaped ventral sucker is similar to that described by Hegazi (1978) and Ahmed (1994). The extension of vitellaria from behind ventral sucker till the posterior end of the body, shape and location of testes in the posterior half of the body, shape and position of ovary and Mehlis' gland are similar to that described by El-Naffar and Khalifa (1975), Hegazi (1978) and Ahmed (1994). The present specimens of *N. ramosum* resemble that of *Echinostoma atrae* that were recovered from black coot *Fulica atra* in Pakistan (Birmani et al., 2008) in general shape and arrangement of internal organs, but *E. atrae* has 38 collar spines and testes are widely separated.

The main differences between echinostomes were observed in the shape, number and arrangement of collar spines, tegumental spines and sensory papillae. The present study gives for the first time the description of *N. ramosum* by using SEM. The shape of collar spines of *N. ramosum* and their number differ from those of *Echinostoma cinetrochis* (Lee et al., 1992), *Echinostoma caproni* (Sotillo et al., 2012) and *Echinostoma revolutum* (Chantima and Wongsawad, 2012), where these species of *Echinostoma* are characterized by presenting 37 spines in their collar and the shape of these spines are cone-shaped, while the arrangement of collar spines of *N. ramosum* is similar to those of *E. caproni* and *E. revolutum*, where the collar spines are arranged in a single row, but in case of *E. cinetrochis* the collar spines are arranged in a zigzag pattern. However, several authors have published different and contradictory studies relative to the arrangement of collar spines

(Kanev et al., 2009).

SEM of excysted metacercaria and adults showed some basic differences in worm topography .The excysted metacercaria is conical shaped ,while the adult is elongated, the ventral sucker of excysted metacercaria is located at about the anterior third of the body, where it located closely in the anterior fifth of the body in the adult worms. The lip of oral sucker in excysted metacercaria, is not well developed, while it is well developed, prominent, and thick in the adult. The ventral sucker showed marked changes in shape in which, the outer rim is smooth, while the inner rim is highly folded in excysted metacercaria, while the outer and inner rims are smooth in the adult.

SEM observations showed that some collar spines retracted into their pockets. The retractions of spines into spinal pockets are in contradictory state in different literature reviews, which tended to depend on the number and arrangement of collar spines (Fried et al., 2009). The mechanisms of spinal extension and retraction were elucidated by Kruse et al. (1992) and Fujino et al. (1994) using different microscopical techniques such as SEM and TEM in *E. trivolvis*. The retraction of a collar spine may result from the relaxation of the muscle bundles associated with the spine (Fujino et al., 1994), and although these authors correlated extended and retracted spines to external factors such as the culture medium, in our case, the reason for the retraction of collar spines in the excysted metacercaria and the adults may be the conditions of fixation procedures for SEM done in this study.

Our SEM study showed that the body tegument of the excysted metacercaria and the adult of *N. ramosum* is devoid of spines, this result is similar to that of *Echinostoma atrae* (Birmani et al., 2008), but dissimilar to those of *E. revolutum* (Fried and Fujino, 1984), *E. caproni* (Ursone and Fried, 1995 and Sotillo et al. 2012), *Echinoparyphium recurvatum* (Lee et al. 1990), *Echinostoma hortense* and *Echinostoma cinetorchis* (Lee et al., 1986, 1992), in which the tegument of anterior body is armed with numerous spines. On the other hand, the highly corrugated surface of the tegument of *N. ramosum* was not observed in the other echinostomatids.

In the present study, two types of sensory papillae on the dorsal surface of the cephalic region of the adult worm were observed, while the other parts of the body of the adult worm are devoid of papillae, this contradicts with *E. revolutum* (Fried and Fujino, 1984) and *E. caproni* (Ursone and Fried 1995), in which abundant papillae were prominent on the lips of both oral and ventral suckers and on the collar peripheral to the oral sucker and differ with *E. hortense* and *E. cinetorchis* (Lee et al., 1986, 1992) in which two types of sensory papillae on oral and ventral suckers, on oral collar and numerous papillae were distributed on the tegument of the ventral surface. The nomenclature of the various types of sensory papillae is different depending on the author, for example, Han et al. (2003) described two types of sensory papillae as types I and II in the echinostomatid *Himasthla alincia*, where as Maldonado et al. (2001), Toledo et al. (2000) and Nakano et al. (2003) used a descriptive nomenclature in different species from the genus *Echmostoma*. The function of the papillae is suggested to be mechanoi, rheo, and chemo-receptors, also the papillae may function as a contact receptor and may have a secretory function (Smales and Blankespoor, 1984). All such functions possibly help in feeding and assessment of the environment (Lee et al., 1987; Fujino et al., 1989). However, further studies regarding the sensory papillae should be undertaken as their shape and distribution are of importance in the differentiation of species within the family Echinostomatidae.

The present study shows numerous papillae on the tegumental surface of the protruded cirrus of *N. ramosum*, these papillae were not observed in the other echinostomes. On the other hand, the cirrus of *E. caproni* had concentric tegumentary folds (Ursone and Fried, 1995), while the tegumental surface of the cirrus of *E. hortense* and *E. cinetorchis* was smooth (Lee et al., 1986, 1992).

In summary, for the first time the ultrastructural features of the tegumental surface of excysted metacercaria and adult worm of *N. ramosum* were described, among these features are the number of collar spines, the restricted distribution of sensory papillae, the corrugated surface of the tegument and the absence of tegumental spines which may be useful as identification criteria for the species *N. ramosum* and even for the genus *Nephrostomum*, and these morphological features were compared with other echinostomatids.

Conflict of interest

The authors declare that they have no conflict of interest.

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