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Light and Scanning Electron Microscopic Studies on *Eustrongylides exciscus* Larvae (Nematoda: Dioctophmida) from *Channa punctatus* Bloch from India

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ABSTRACT

Fourth stage larvae of *Eustrongylides exciscus* were recovered from the abdominal cavity, musculature and ovaries of *Channa punctatus* (prevalence 24%, intensity 2 to 18 per fish). Light and Scanning Electron Microscopy (SEM) were conducted to demonstrate the surface anatomy of the worm. The worm is slender, elongated and cylindrical. The mouth is slit-like, surrounded by two lateral rows of somatic papillae, cephalic end was conical with 12 labial papillae arranged in two circles of 6 papillae each. Scanning electron microscopy on attachment structures revealed that the inner papillae had narrow bases and spine-like apices, the outer ones had wide bases and nipple like apices encircled by a conspicuous ring and embedded in the body wall. The body of the worm was striated and grooved assisting flexibility to the worm during movement. Attempts to develop the larvae in ducks *Anas platyrynchas* by feeding were unsuccessful up to 17 days post infection suggesting that ducks were not the final hosts of the worm. *Channa punctatus* serves as a paratenic host for *Eustrongylides*.

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INTRODUCTION

Eustrongylides is a nematode causing Eustrongylidosis. Due to its large size, it is commonly known as the Big Red Worm. *Eustrongylides* infection has been reported from various regions of the world including North and South America, Europe, Eastern Africa, China and Turkey (Paperna, 1974; Moravec *et al*., 2003; Salgado-Maldonado *et al*., 2004; Saraiva *et al*., 2006; Saffari *et al*., 2007). These nematodes have attracted attention because they are widely distributed in various geographical regions and exhibit a great potential for transmission and pathogenicity. However, most of the previous research on various species of the genus has been limited to faunal investigation in different geographical sites and host species, as well as the pathology in their host (Measures, 1988a, b; Asakawa *et al*., 1997; Junker *et al*., 2006; Mihalca *et al*., 2007). Information on the life cycle of *Eustrongylides* is limited. It is very complicated and is completed in different stages. In fishes, the parasites are conspicuous as long, red, coiled larvae located in the body cavity or embedded in the muscles (Mitchum, 1995; Overstreet, 2003). In humans who have consumed raw or uncooked fish, *Eustrongylides* produces gastritis and intestinal perforations (Deordorff and Overstreet, 1991; Cole, 2009).

There are a few reports of *Eustrongylides* from India. Ali (1971) reported a new species, *E. indicus* from an Indian bird. However, Kalyankar (1974) for the first time described the larval stage of the worm in the body cavity of *Macrones seenghala* from Nanded. The pathological effects caused by *Eustrongylides* larvae on serum LH level and histology of gonads of *Clarias gariepinus* were reported by Mir *et al*. (2012) recording a significant decrease (50%) in the gonadotropin hormone (LH) in the blood. Histopathological studies of testes revealed disorganized testicular structure, germinal epithilium was disrupted and reduction in spermatozoa was reported by the authors. Histology of infected ovaries showed the appearance of interfollicular spaces, degeneration of follicular wall tissues and their egg envelops were ruptured. Jaiswal *et al*. (2013) reported the ecological dynamics of *E. tubifex* in silver whiting, *Sillago sihama* from the central west coast of India, Goa.

Morphological details of the parasite examined under light microscopy are significant for morphological studies but in fishes, *Eustrongylides* are present in their larval condition, therefore identification of the reproductive structure of the parasite is not possible. SEM represents an important tool for studying the detailed surface topography of the parasite and its ability to provide three

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dimensional images with high magnification allow a better understanding of the spatial relationships among surface structures. This technique is therefore acquiring importance for validating species and demonstrating differences between populations or races (Gibbons, 1986; Cynthya *et al*., 2012). The shape, number, and distribution of papillae on the cephalic end are important characters used in taxonomy and classification. The development of the larvae in the definitive host provides information on the life cycle pattern. Therefore, the present study was designed to study the morphological and topographical characteristics of *Eustrongylides* larvae collected from *Channa punctatus* and further attempts to develop the larva in domestic ducks (*Anas platyrhynchos domestica* L.) in order to test their viability as potential hosts of the worm.

MATERIALS AND METHODS

Collection and maintenance of host

During spring 2012-2013, 250 live samples of the fishes (*Channa punctatus* Bloch, 1793 Perciformes: Channidae) weighing 25-45 g were collected from local ponds, river Ramganga of Bareilly, Uttar Pradesh, India (28.35ºN; 79.42º E). The fishes were transported to the Centre of Excellence laboratory of M.J.P. Rohilkhand University in large containers and maintained in aquaria under proper aeration. They were fed on commercial pellets (Toya fish food containing fish and soya bean meal, wheat and rice flour and vitamins A, C, D_3 and E till sacrificed. They were treated in accordance with the guidelines of the local ethics committee.

Light microscopic preparation

Prior to sacrifice, fishes were anesthetized and abdominal cavity, digestive tract and other organs were thoroughly examined for the presence of *Eustrongylide*s larvae by examining the tissues under a Magnus Zoom Stereo Trinocular microscope with Magnus Image Projection System, TS100-F (LED type). The parasites collected were fixed in 70% ethanol or 4% formaldehyde. They were instantly examined by clearing in lactic acid, optically examined microscopically by clearing in glycerine or cleared in pure glycerine or lactophenol (Kakar and Bilqees, 2016), optically and examined microscopically. by clearing in glycerin. Drawings were made and photographs taken under Olympus BX 53 microscope with DIC attachment, digital camera and CELLSENS imaging software system.

Scanning electron microscopic preparation

For SEM analysis, the specimens were washed with 0.1 M phosphate buffer (pH 7.2), fixed in 2% cold glutaraldehyde buffered with 0.1 M phosphate buffer for 15-20 hours at 4˚C in a refrigerator, washed 2-3 changes in 0.1 M phosphate buffer, post fixed in 1% osmium tetra oxide for 1 hour, dehydrated through graded series of ethanol (70% to 100%) for 10 minutes each at room temperature and dried to critical point. Specimens were glued to metal specimen stubs with double sided tape, rotary-coated with gold mixed with palladium using a sputter coater (NeoCoater 100-240V) for 30 sec, examined in a scanning electron microscope (Neo JCM-6000) at an acceleration voltage of 15 kV.The results were recorded, saved on computer and visualized on LCD screen.

Life cycle

The life cycle is indirect, requiring two intermediate hosts. It commences with the egg stage which are always inside a poop. The first stage larva develops within the eggs that are shed in bird faeces. The eggs fall in water and can live for 2⅓ year before being eaten up by freshwater oligochaetes/aquatic worms serving as the first intermediate host. Eggs hatch within the oligochaetes and convert into the second and third stage larva formed inside the eggs. To continue its life cycle, the worm needs a fish to eat the aquatic worm. The larvae remain inside the fish body serving as the second intermediate host and the third stage larvae become encapsulated on the internal surface areas of the fish and develop into the infective fourth-stage larvae till eaten by predatory birds, the final hosts. The cycle maybe elaborated by predatory fish feeding on the infected fish thereby serving as paratenic/transport hosts and these ultimately being fed upon by birds. Once inside a bird, the worm can complete its life cycle. Herons, egrets and sometimes eagles act as the final hosts. In the bird's stomach, the larva becomes an adult, attains maturity and is capable of shedding eggs in about 10-17 days post infection.

In fish, unencysted larvae of these parasites migrate under the skin and in the muscles causing extensive inflammation and necrosis. Encystation occurring in the viscera namely the liver, spleen or gonads causing severe pathologic changes in the adjacent tissue (Paperna, 1974).

Attempts to develop Eustrongylides *(IV stage larva) in domestic ducks (*Anas platyrynchas*)*

One year old domestic ducks, *Anas platyrynchas* (n=6) were purchased from Bareilly market, maintained outdoors in large metal cages and were provided fresh water and duck feed daily during experimentation.

Ducks were randomly separated into two groups, 4 in the test group and two in the control group. Ducks in the test group were fed with duck feed and 7 *Eustrongylides* larvae

each collected from the intestinal tract of *C. punctatus* whereas those of the control group were fed only on duck feed. From day 2 post infection (PI) onwards, the faecal sample of each duck was examined. On day 17 PI, the remaining ducks in both groups were killed and all internal organs and body cavity of the ducks were examined for the presence of *Eustrongylides*.

Fig. 1. L4 dissected *Channa punctatus* showing *Eustrongyloides tubifex* lying in the body.

RESULTS

The infected fish showed bloody patches on its surface. On autopsy, the gastrointestinal tract of *C. punctatus* revealed the presence of the highly coiled nematode parasite in the abdominal cavity, musculature and ovaries (Fig. 1). The prevalence was 24% and the number of parasites per fish ranged from a 2 to 18 (Fig. 2). Parasite body tapering with dense transverse striation of cuticle, anterior end pink, remaining part of body red with deepest colouration in mid-anterior part of body. The reproductive organs of the parasite were not developed, based on its morphology, the parasite is identified as IV stage larva of *Eustrongylides excisus*.

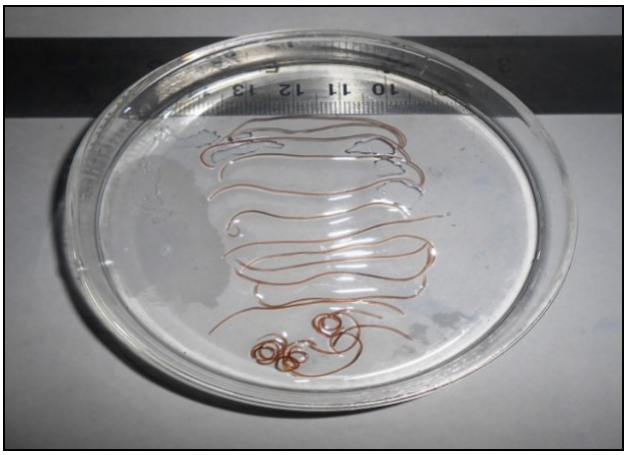

Fig. 2. L4 *Eustrongylides tubifex* isolated in a petri dish from a single fish.

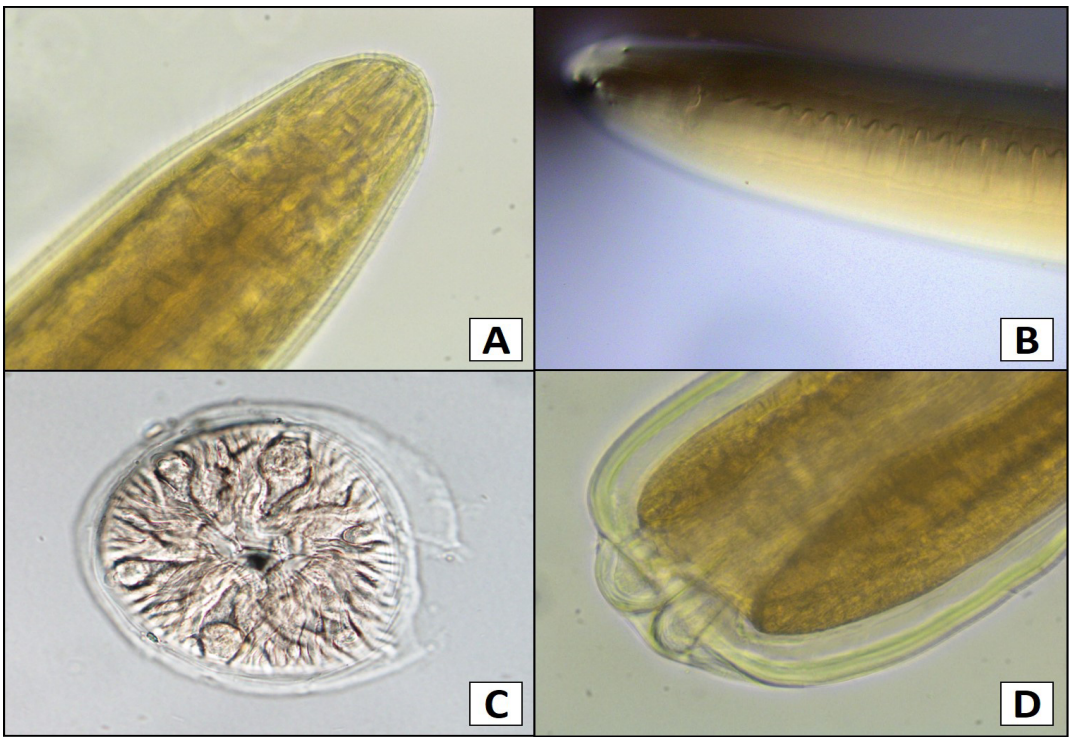

Fig. 3. Light microphotographs of L4 *Eustrongylides tubifex*: **A,** anterior end showing tapering end; **B,** anterior end showing papillae under fine adjustment; **C,** *en face* view showing arrangement of papillae; **D,** posterior blunt end.

Light microscopy studies on the worm and SEM on attachment structure (papillae) were performed. All measurements are given in micrometers.

Light microscopy

The larvae were studied under Olympus BX 53 microscope with DIC attachment, digital camera and CELLSENS imaging software system. The parasite was bright red in colour, length of body was 38.5-45.8 mm, maximum width 3.06 mm, oesophagus 10.20 mm. Anterior end somewhat narrow. Three cuticle layers were visible at the anterior and posterior extremities of the worm, apparently the second and third stage cuticles were retained (Fig. 3A-D).

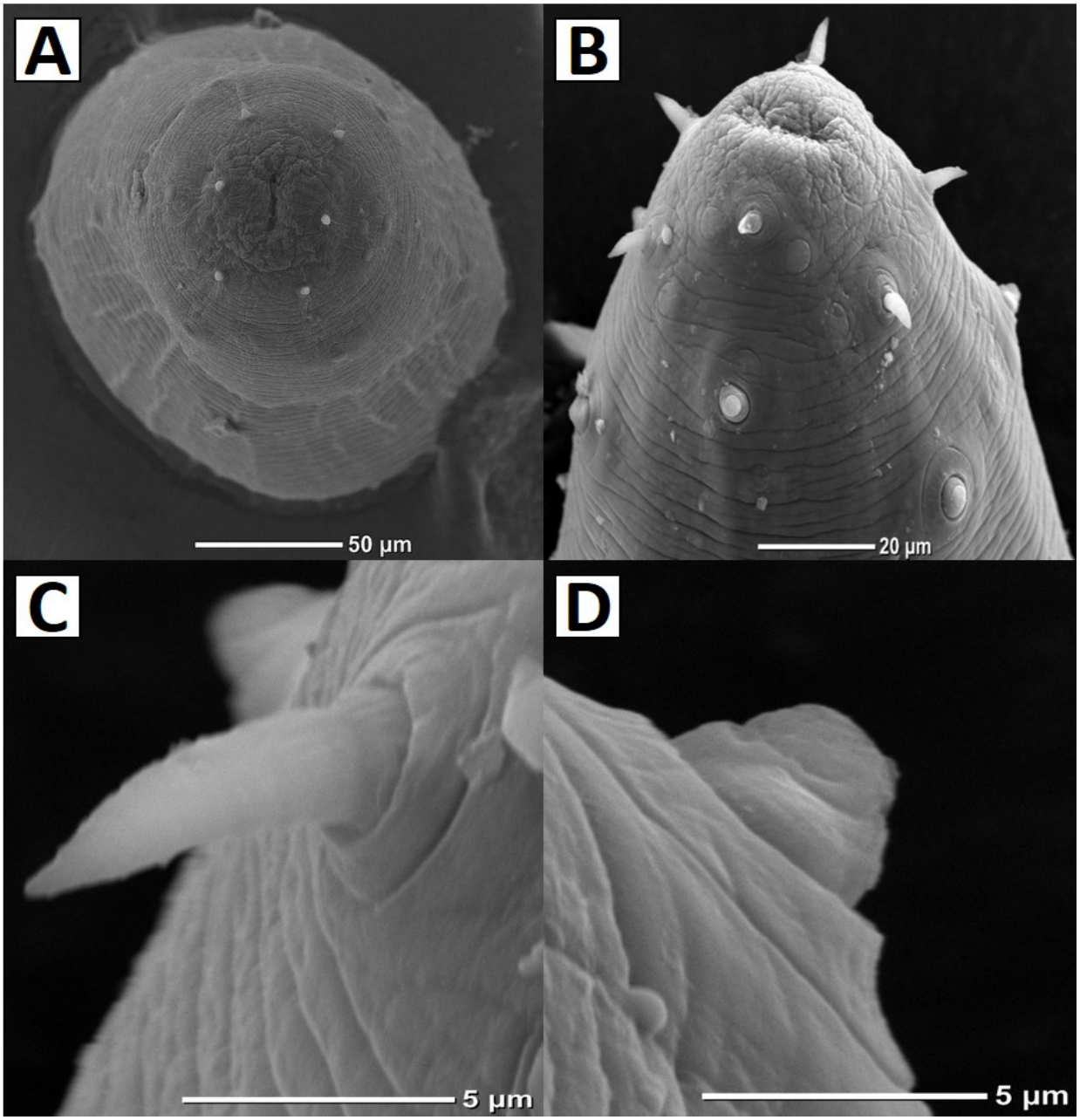

Fig. 4. Scanning electron micrographs of L4 *Eustrongylides tubifex*: **A,** *en face* view showing slit-like mouth (arrow) and number of papillae; **B,** anterior end showing arrangement of papillae; **C,** inner papilla showing narrow base and spine-like apex; **D,** outer papilla having wide base and nipple-like apex.

Scanning electron microscopy

SEM revealed two lateral rows of somatic papillae extending along the body. The cephalic end was conical and showed 12 labial papillae arranged in two circles of 6 papillae each, including 2 lateral, 2 sub ventral, and 2 sub dorsal (Fig. 4A). The inner papillae had narrow bases and spine-like apices (Fig. 4B, C), whereas the outer ones had wide bases and nipple like apices (Fig. 4D). The latter were encircled by a conspicuous ring and securely embedded in the body wall (Fig. 5A). Just above these papillae, sensilla were apparent which are probably sensory in nature (Fig. 5B). The body of the worm was striated (Fig. 5C) and under high magnification, appeared to be grooved providing flexibility to the worm during movement (Fig. 5D).

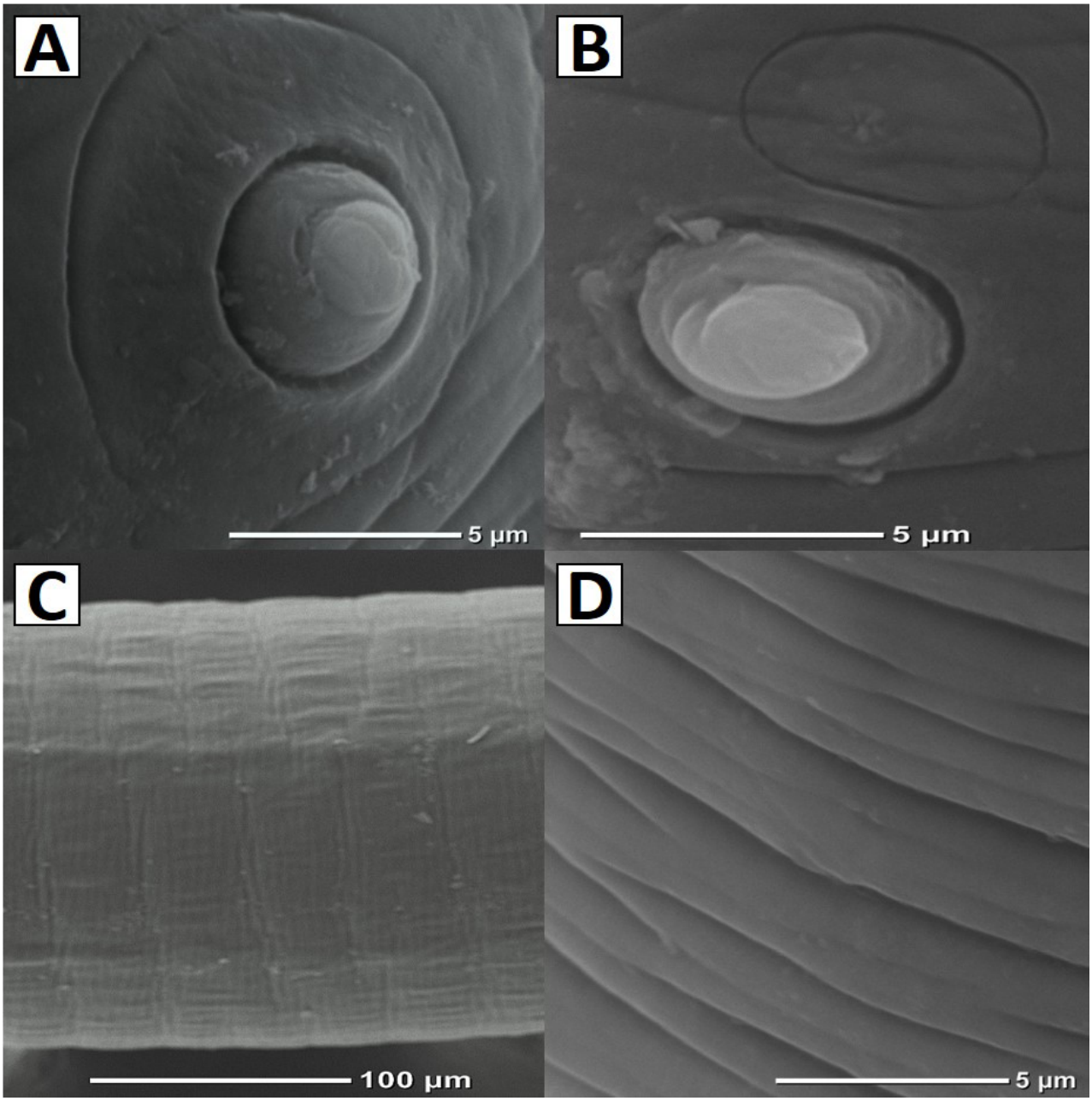

Fig. 5. Scanning electron micrographs of L4 *Eustrongylides tubifex*: **A,** surface view of outer papilla encircled in a ring; **B,** sensillae situated above the outer papilla; **C,** body showing striations; **D,** striations under higher magnification (note the grooves and incomplete margins).

Comments

Eustrongylides is one of the four genera of adenophorean nematodes belonging to the superfamily Dioctophymatoidea, family Dioctophymatidae Railliet, 1915, family Eustrongylinae Chitwood and Chitwood, 1937 (Anderson *et al*., 2009). According to Measures (1988b), only two valid species of the genus *Eustrongylides* Jagerskiold, 1909, *E. ignotus* Jagerskiold, 1909 and *E. tubifex* (Nitzsch and Rudolphi, 1819) Jagerskiold, 1909, are known to occur in the New World; the definitive hosts of the former are piscivorous birds belonging to the orders Ciconiiformes and Pelecaniformes and that of the latter are Gaviiformes, Anseriformes, Ciconiiformes and Podicipediformes birds.

Three species of *Eustrongylides* are accepted as valid taxa: *E. tubifex* Jagerskiold, 1909, *E. ignotus* Jagerskiold, 1909 and *E. excisus* Jagerskiold, 1909 (Xiong *et al*. 2009). The labial papillae of the inner circle are larger than those of the outer circle in *E. ignotus* and *E. excisus* whereas the reverse is true for *E. tubifex*. In *E. ignotus* males, the outer perimeter of caudal sucker has a wide cuticular hem and the inner perimeter lacks cuticular projections, a ventral cleft is absent in the caudal sucker whereas in *E. excises* males, the outer perimeter of the caudal sucker has a reduced cuticular hem, the inner perimeter of caudal sucker has a row of cuticular projections and a deep ventral cleft is present on the caudal sucker (Measures, 1988b). However, as the detected worms were larvae, therefore, these characters could not be ascertained and further transmission experiments are recommended.

DISCUSSION

Earlier studies on larval *Eustrongylides* mainly focused on the number and arrangement of papillae which were used as diagnostic characters. However, the present studies on scanning electron microscopy indicated that the inner papillae had narrow bases and spine-like apices, the outer ones had wide bases and nipple like apices encircled by a conspicuous ring and embedded in the body wall. The body of the worm was striated and grooved assisting flexibility to the worm during movement. These observations are an advancement on the earlier reported light microscopic studies.

The present SEM studies on *Eustrongylides* larvae would be useful in future studies as this is the first such report from India and the details of the larva described herein would assist future related workers.

The present survey shows that larval nematodes frequently parasitize fishes of Rohilkhand, Uttar Pradesh, India. Often it is difficult to identify larvae to species level in fish without feeding them to birds. Moreover, the degree of host specificity of nematode larvae to their fish hosts has been regarded to be lower than that of adult nematodes and have been reported to occur in phylogenetically distant fish orders (Moravec, 1994; Moravec *et al*., 1995). However, during the present studies, the larvae were specific to *Channa punctatus* whereas *C. striatus, Heteropneustes fossilis* and *Clarias batrachus* collected from the same sites did not show any infection. Numerous larvae present in fish hosts maybe harmful (Moravec *et al*. 1995).

Larvae are quite large in size and they often infect fishes with high intensity, therefore the hosts abdomen is bloated (Yanong, 2011). Consuming infected fish, predators can be infected and serve as paratenic host when they are fed upon by birds (Cole, 2009). According to Moravec *et al*. (1995) larvae of the genus *Eustrongylides* belong to the group of the most pathogenic parasites. By the character of their cephalic papillae the larva of the present material resemble *E.excisus*, but their possible appurtenance of this species should be confirmed experimentally. Our attempts to infect domestic ducks (*Anas platyrynchos*) with *Eustrongylides* from *C. punctatus* were unsuccessful. suggesting that ducks are not suitable definitive hosts for this species.

Eutrophication and warm water temperatures (20-30°C) are optimum conditions for the survival of the parasite. Water quality is an important factor as infection in fish is highest where external sources of nutrients or thermal pollution alter the natural environment. Therefore, to check infection, water quality should be improved relative to land-use practices and waste water discharges (Cole, 2009).

The occurrence of nematode larvae in fish is not obligatory for completing its life cycle, however, they appear to be important ecologically by acting as paratenic hosts in causing infection in the definitive host.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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