First Report on Histochemical Studies of Egg Shell of Opisthorchis pedicellata (Verma 1927) (Digenea: Opisthorchiidae), Shows the Presence of Basic Proteins, Protein and Phenols

Sita Yadav, Ratnesh Kumar Singh, Madhu Tripathi and A. M. Saxena

Research Journal of Agricultural Sciences
An International Journal

P- ISSN: 0976-1675
E- ISSN: 2249-4538

Volume: 13
Issue: 03

Res. Jr. of Agril. Sci. (2022) 13: 876–880

CARAS

Res. Jr. of Agril. Sci. (May-Jun 2022)
13(3): 876–880

ISSN: 0976-1675 (P)
ISSN: 2249-4538 (E)

www.rjas.org

Full Length Research Article

First Report on Histochemical Studies of Egg Shell of *Opisthorchis pedicellata* (Verma 1927) (Digenea: Opisthorchiidae), Shows the Presence of Basic Proteins, Protein and Phenols

Sita Yadav[1], Ratnesh Kumar Singh*[2], Madhu Tripathi[3] and A. M. Saxena[4]

Received: 03 Apr 2022 | Revised accepted: 18 Jun 2022 | Published online: 22 June 2022

A B S T R A C T

In recent years, the development of histochemical approaches has been very fast. Helminth embryos have a distinct shell that is generally referred to as egg-shell. A total of 15 Rita rita (Hamilton 1822) were collected from the Unnao fish market, and total of 45 *Opisthorchis pedicellata* were recovered from gall bladder of the host. Different results of histochemical observation found in this study of egg shell formation of *O. pedicellata*. Aqueous bromophenol blue test shows the moderate orangish blue colour, while Ninhydrin Schiff test shows strong pinkish red colour, both these two tests indicate the presence of basic protein. For the presence of protein, we test the Xanthoproteic and Biuret Test which shows the strong orange and blue colour respectively. For the presence of phenols, Argentaffin, Chromaffin reaction, Sodium Iodate test and Ferric Chloride test were showed the dark brown, brown and dull green colour respectively.

***Key words*:** Histochemical tests, *Rita rita*, Egg shell, Opisthorcis, Protein, Phenols

Opisthorchis pedicellata Verma (1927) was described from a siluroids *Rita rita* Hamilton, 1822. The worms are morphologically comparable in terms of sucker ratio, cirrus sac location and extension of vitelline follicles, and gonad configuration. The embryo of platyhelminths possess unique characteristic shell which commonly knowns as egg-shell. It is most fascinating and interesting characteristic attribute which protects the embryo. Embryo faces many hazards such as desiccation, biological hazards, chemical hazards during the course of development, at that time egg shell acts as an insurance. Mehlis gland secretes lipoproteinous substances which is poured off into the lumen of ootype complex that forms an outer membrane around the whole egg. At the same time globules secreted by vitelline cells acts as a precursors of shell materials. These precursor molecules now coalesce to form a semi liquid shell inside the lipoprotein layer which undergoes tanning process, as a result of which it becomes tough and resistant. Mehlis' glands play important role in egg shell formation. Vitelline glands cells exist in different stages of their development and their roles also varies with their development.

* **Ratnesh Kumar Singh**
✉ ratneshsingh1929@gmail.com

[1-4] Department of Zoology, University of Lucknow, Lucknow - 226 007, Uttar Pradesh, India

Considerable amount of work has been carried out on the chemical composition of egg shell, roles of Mehlis glands, ootype complex and vitelline glands. Concluding the most of work, almost all authors reported that egg shell is made up of resistant tanned protein which is subsequently covered by thin envelop of lipoprotein. Vitelline glands produce precursors of egg shell formation while roles of Mehlis' glands also important in egg shell formation. Egg- shell of almost all trematodes consists of tanned protein [12] but some exceptions are also there such as in amphistomes keratin may be the major shell protein [9] while in *Orchispirium heterovitellatum*, elastin may be the major constituent [7] Precursors of the quinine tanning (Phenols, Phenolase and proteins) were histochemically localized in the globules of vitelline cells [3] and later confirmed by several Indian workers [10]. The reaction of quinone and tannin takes place by O-diphenolic acid is oxidised to O-quinone then alteration of –OH and -NH_2 radicals in tyrosine rich proteins, then after moieties of tryrosine are oxidised into O-diphenolic acid and then into quinine. The quinine now reacts with free -NH_2 groups in adjacent protein chain and establishes strong covalent bond. In this way it forms cross linked highly strong and stable protein. In due course of time some conflict arose in egg shell composition, chemical composition of paramphistomes egg shell consist of keratin due to difference in constituent basic protein [8-9] and further pointed out that there were no traces of Phenol, Phenolase etc. in the vitelline cells thus, it uses some other process for stabilization. Therefore, there is need of further study to

investigate the all-digenetic trematodes egg shell biochemistry because only limited studies are available till now. Therefore, the aim of this work to full fill some gap that can give some more information about other trematodes egg shell biochemistry.

MATERIALS AND METHODS

Freshwater fish were collected from fish market, Unnao for the collection of trematodes. Fishes were identified with the help of keys "Fishes of U. P. and Bihar" [13]. The fishes were dissected and their alimentary canal, liver, kidney, gall bladder, heart and gills were thoroughly examined and kept in physiological saline (0.87%). Live specimens of parasites were collected from the intestine of host and kept in normal saline. Recovered parasites were fixed in appropriate fixative for 24 – 48 hr. Parasites were dehydrated in ascending grade of alcohol, cleared in xylene and embedded in molten wax. Microtomy section of about 6 to 8 µm were prepared for histochemical analysis.

Reagents required

Aqueous Bromophenol Blue (0.1%): 200 mg bromophenol blue dissolved in 200 ml of distilled water.

0.5 % Ninhydrin: 1 gm ninhydrin dissolved in 200 ml absolute alcohol.

Schiff reagent: 1 gm basic fuschin powder dissolved in 200 ml of boiling water; shaken for 5 minutes and cooled to 50°C.

This solution was filtered and 20 ml of 1N HCl (89 ml concentrated HCl of specific gravity 1.18 + 911 ml of distilled water) was added, cooled to 25°C. Then after 1 gm of sodium or potassium metabisulfite ($Na_2S_20_5$ or $K_2S_20_5$) was added. The solution was kept, in dark, for 14 - 14 hrs) 2 gm of activated charcoal was added to the solution and shaken for 1 min. filtered, and filtrate was kept in dark at 0 – 4°C in a refrigerator (Solution was left for 15 – 25 min to reach at room temperature before use).

KOH (10%): 5 gm KOH dissolved in 50 ml distilled water.

$CuSO_4$ (1%): 500 mg of $CuSO_4 .5H_2O$ crystal dissolved in 50 ml of distilled water.

Ammonical silver nitrate: concentrated ammonia was added to 0.1% $AgNO_3$, prepared by dissolving 100 mg $AgNO_3$ in 100 ml distilled water.

Sodium iodate (10%): 10 gill of sodium iodate was dissolved in100 ml of distilled water.

0.5 N ferric chloride: 8.11 gm of $FeCl_3$ was dissolved in 100 ml of distilled water.

Test for proteins

Xanthoproteic Test (Pearse, 1953): Paraffin sections were deparaffinised in xylol and brought into water through descending grade of alcohol (absolute, 90%, 70%, 50% and 30%), the sections became orange as they were treated with concentrated HNO_3, washed with distilled water, immersed in dilute ammonia solution and mounted in glycerine. Development of orange colour show the presence of proteins.

Biuret Test (Srivastava, 1978): Paraffin sections were deparaffinised in xylol and brought into water through descending grade of alcohol (absolute, 90%, 70%, 50% and 30%), the sections were immersed in 10% KOH solution. Few drops of 1% $CuSO_4$ solution was added. During this process, the solution was stirred vigorously. Development of blue colour shows the presence of proteins.

Test for phenols

Argentaffin reaction (Bell and Smyth, 1958): Paraffin sections were deparaffinised in xylol and brought into the water through descending grades of alcohols (absolute, 90%, 70%, 50% and 30%) and treated with ammoniacal silver nitrate, drop by drop until the precipitate dissolved, for 20 – 24 hrs, washed in ammonia for two hours, dehydrated through ascending grades of alcohol (30%, 50%, 70%, 90% and absolute). cleared in xylol and mounted in Canada balsam. Development of dark brown colour shows the presence of phenols.

Chromaffin reaction (Lewis, 1962): Paraffin sections were deparaffinised in xylol and brought into the water through descending grades of alcohols (absolute, 90%, 70%, 50% and 30%) and treated with a mixture of 10 volumes of 5% potassium dichromate (5 gm $K_2Cr_2O_7$, dissolved in 100 ml of distilled water) for 30 min and then transferred to a freshly prepared mixture of 10 volumes of 5% $K_2Cr_2O_7$) 1 volume of 5% potassium chromate, 2 volumes of formaldehyde and 7 volumes of distilled water for 12 to 24 hrs. The sections were washed in running water and further in distilled water, dehydrated through ascending grades of alcohol (30%, 50%, 70%, 90% and absolute), cleared in xylol and mounted in Canada balsam. Development of brown colour shows the presence of phenols.

Sodium Iodate test (Srivastava, 1978): Paraffin sections were deparaffinised in xylol and brought into the water through descending grades of alcohols (absolute, 90%, 70%, 50% and 30%) and with 10% sodium iodate for 2 hours, then treated with 10% formalin for 24 hrs, dehydrated through ascending grades of alcohol (30%, 50%, 70%, 90% and absolute), cleared in benzene and mounted in DPX. Development of brown colour shows the presence of phenols.

Ferric Chloride test (Srivastava, 1978): Paraffin sections were deparaffinised in xylol and brought into the water through descending grades of alcohols (absolute, 90%, 70%, 50% and 30%) and treated with few drops of 0.5 N ferric chloride. Development of dull green colour shows the presence of phenols.

Test for basic protein

Aqueous Bromophenol Blue test (Maiza, Brewer and Alfert, 1953): Paraffin sections were deparaffinised in xylol and brought into the water through descending grades of alcohols (absolute, 90%, 70%, 50%, and 30%). The sections were stained with 0.1% Aqueous Bromophenol Blue for 10 min. The section rinsed in acetic acid solution (0.5%) for 20 min then after in tap water for 3 min and then with distilled water. The sections were dehydrated in ascending grades of alcohol (30%, 50%, 70%, 90% and absolute) and counter stained by Orange – G (1%), cleared in xylol and mounted in DPX. Development of orangish blue colour show the presence of basic proteins.

Ninhydrin Schiff test (Pearse, 1968): Paraffin sections were deparaffinised in xylol and brought into the water through descending grades of alcohols (absolute, 90%, 70%, 50%, and

30%). The sections were treated with 0.5% ninhydrin for 16 – 20 hrs at. 37°C, washed in running water for 2 – 5 minutes and treated with Schiff reagent. The sections were washed in running water for 10 min and dehydrated through ascending grades of alcohols (30%, 50%, 70%, 90% and absolute), cleared in xylol and mounted in Canada balsam. Development of Pinkish red colour shows the presence of basic proteins.

RESULTS AND DISCUSSION

A total of 15 *Rita rita* (Hamilton, 1822) were collected from the Unnao fish market, and total of 45 Opisthorchis pedicellata were recovered from gall bladder of the host. The prevalence was 100%, while the mean intensity and index of infestation was three.

The result of histochemical observation found in this study of egg shell formation of *O. pedicellata* are given in (Table 1). Aqueous bromophenol blue test shows the moderate orangish blue colour (Fig 1), while Ninhydrin Schiff test shows strong pinkish red colour (Fig 2), both these two tests indicate the presence of basic protein. For the presence of protein, we test the Xanthoproteic and Biuret Test which shows the strong orange (Fig 3) and blue colour (Fig 4) respectively. For the presence of phenols, Argentaffin, Chromaffin reaction, Sodium Iodate test and Ferric Chloride test were showed the dark brown (Fig 5), brown (Fig 6-7) and dull green (Fig 8) respectively. The immature and young eggs were strongly positive to all the above-mentioned histochemical tests, but as they start maturing, their ability to stain with argentaffin, chromaffin, sodium iodate and ferric chloride tests start reducing indicating the absence of phenols. When the eggs matured their precursor, phenols convert into quinones. The precursor of egg-shell of *O. pedicellata* are protein, basic protein and phenols which shown in our results (Table 1). Therefore, the result of current study reveals that's the egg-shell of *O. pedicellata* is made up of quinone tanned.

Table 1 Histochemical test on egg-shell of *Opisthorchis pedicellata* shows the presence of basic proteins, protein and phenols

S. No.	Histochemical Test	Reacting substances	Presence of colour	Reaction strength
1	Bromophenol Blue Test	Basic proteins	Orangish blue	+
2	Ninhydrin Schiff test	Basic protein	Pinkish red	+
3	Xanthoproteic Test	Proteins	Orange	++
4	Biuret Test	Protein	Blue	++
5	Argentaffin reaction	Phenols	Dark brown	++
6	Chromaffin reaction	Phenols	Brown	+
7	Sodium Iodate test	Phenols	Brown	++
8	Ferric Chloride test	Phenols	Dull green	++

+Moderate, ++Strong

The histochemical analysis of egg shell of *O. pedicellata* was performed an egg collected from freshwater fish *R. rita*. Eight tests were applied to find out the presence of quinone tanning system in egg shell of trematode [12]. The result of current study is supported by Stephenson, Smyth, Guilford, Hanumantha Rao, Ebrahimzadeh, Coil and Srivastava and Gupta [15, 11, 5, 6, 2, 1, 14]. However, many workers reported that's no polyphenol oxidase activity occurred in trematodes such as: *Gorgoderina sp*., *Bucephaloides gracilescens*, *Schistosoma mansoni* and *Protoeces subtenuis* [4, 11] but the egg shell of *O. pedicellata* consist of polyphenol oxidase activity which convert phenol in to quinone.

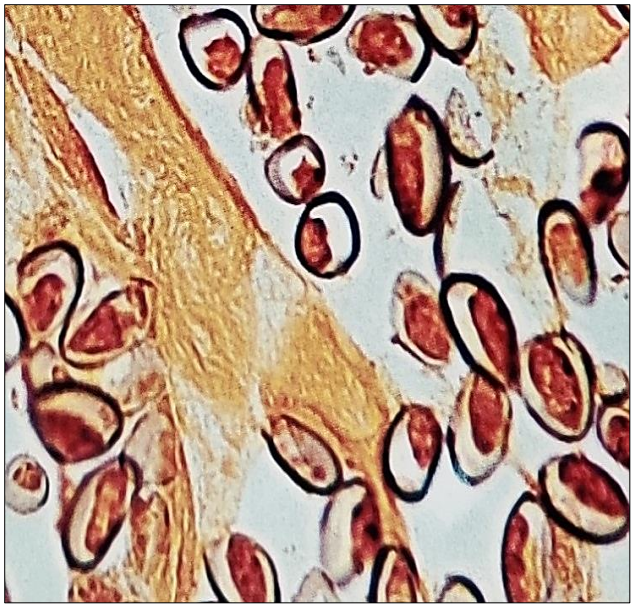

Fig 1 Longitudinal section of *Opisthorchis pedicellata*, orangish blue colour showing presence of basic protein with Aqueous Bromophenol Blue test

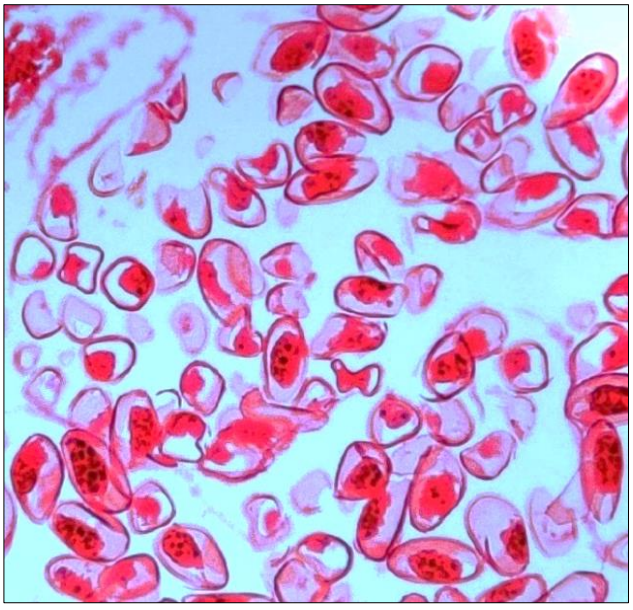

Fig 2 Longitudinal section of *Opisthorchis pedicellata*, pinkish red colour showing presence of basic protein with Ninhydrin Schiff test

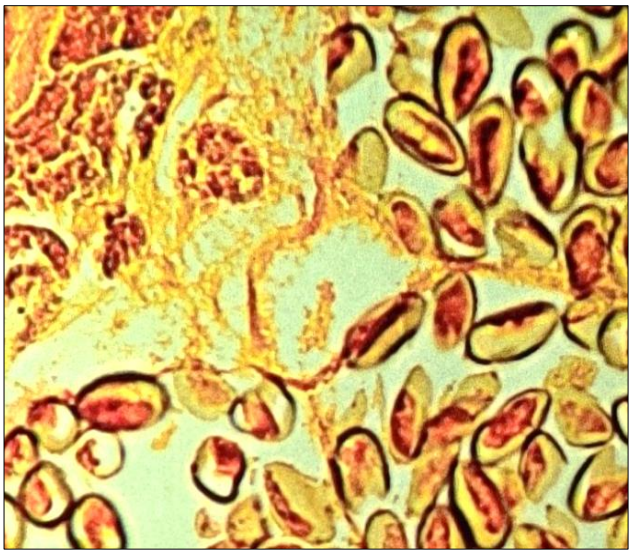

Fig 3 Longitudinal section of *Opisthorchis pedicellata*, orange colour showing presence of protein with Xanthoproteic Test

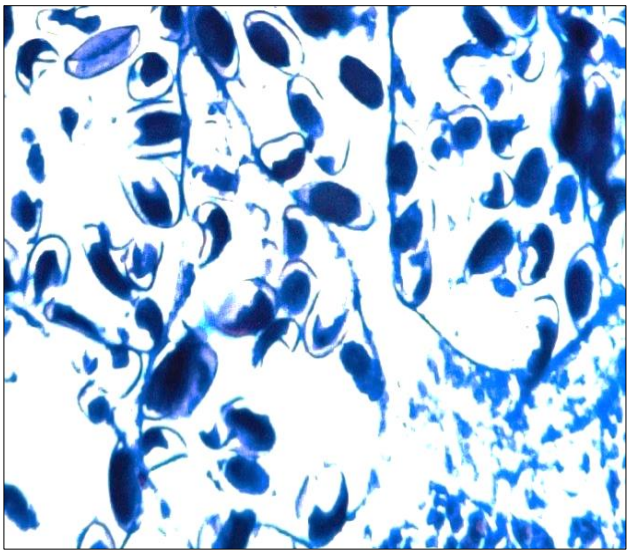

Fig 4 Longitudinal section of *Opisthorchis pedicellata*, blue colour showing presence of protein with Biuret Test

Fig 5 Longitudinal section of *Opisthorchis pedicellata*, dark brown colour showing presence of phenols with Argentaffin reaction

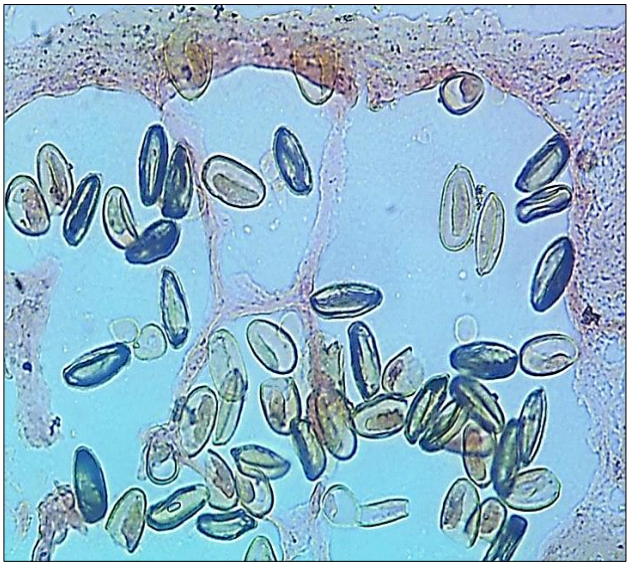

Fig 6 Longitudinal section of *Opisthorchis pedicellata*, brown colour showing presence of phenols with Chromaffin reaction

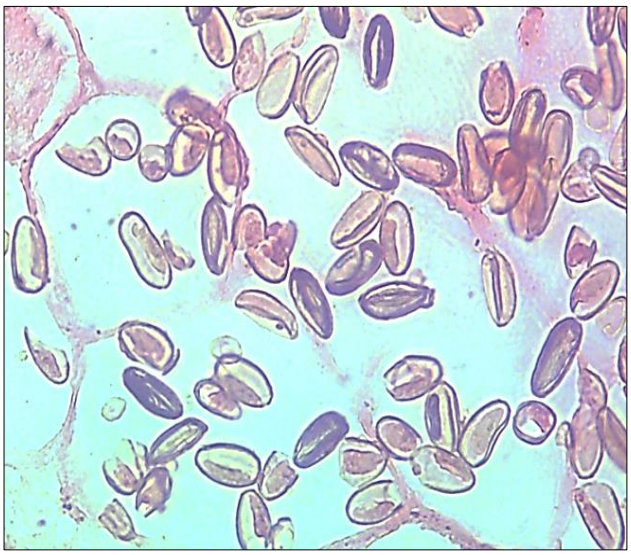

Fig 7 Longitudinal section of *Opisthorchis pedicellata*, brown colour showing presence of phenols with Sodium Iodate test

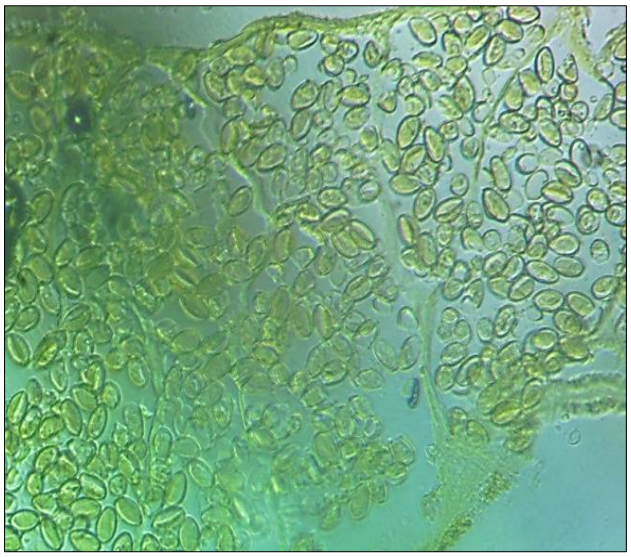

Fig 8 Longitudinal section of *Opisthorchis pedicellata*, dull green colour showing presence of phenols with Ferric Chloride test

The Argentaffin, Chromaffin reaction, Sodium Iodate test and Ferric Chloride test showed the presence of phenol in the egg shell of *Opisthorchis pedicellata* that's supports the quinone tanned system. Madhavi [8] pointed out on the basis of

her histochemical test that egg -shell of several trematodes differs from fundamental nature such as quinone tanned system. She reported that trematodes possess two different mechanisms for stabilizing the egg-shell protein. Madhavi [8] observed that phenolase were absent in amphistomes, there was another keratin type of seleroprotein that is stabilized by disulphide linkages. Srivastava and Gupta [14] reported that is *Isoparorchis hypselobagri* egg shell is colourless and transparent at all stages but both phenols and phenolase are present in the vitelline cells. On the basis of the result of Srivastava and Gupta [14] it is found that mere physical appearance of the egg-shell is not indicative of the presence or absence of phenols and phenolase as suggested by Madhavi [8-9]. Thus, amphistomes egg shell does not follow general pattern and has keratinized egg-shell.

CONCLUSION

The result of histochemical observation found in this study of egg shell formation of *Opisthorchis pedicellata*. Aqueous bromophenol blue test shows the moderate orangish blue colour, while Ninhydrin Schiff test shows strong pinkish red colour, both these two tests indicate the presence of basic protein. For the presence of protein, we test the Xanthoproteic and Biuret Test which shows the strong orange and blue colour respectively. For the presence of phenols, Argentaffin, Chromaffin reaction, Sodium Iodate test and Ferric Chloride test were showed the dark brown, brown and dull green respectively. The immature and young eggs were strongly positive to all the above-mentioned histochemical tests, but as they start maturing, their ability to stain with argentaffin, chromaffin, sodium iodate and ferric chloride tests start reducing indicating the absence of phenols. When the eggs matured their precursor, phenols convert into quinones. The precursor of egg-shell of *Opisthorchis pedicellata* are protein, basic protein and phenols which shown in our results. Therefore, the result of current study reveals that's the egg-shell of *Opisthorchis pedicellata* is made up of quinone tanned.

Conflict of interest

Authors declare no competing interests.

LITERATURE CITED

1. Coil WH. 1965. Observations on egg-shell formation in *Hydrophitrema gigantica*, Sandars, 1960 (Hemiuridae: Digenea). *Z. Parasitenk* 25: 510-517.
2. Ebrahimzadeh A. 1966. Histologische Untersuchungen tiber den Feinbau des Oogenotop bei digenen Trematoden. *Ztschr. Parasitenk* 27: 127-168.
3. Erasmus DA. 1973. A comparative study of the reproductive system of the mature, In mature, and 'unisexual' female *Schistosoma mansoni*. *Parasitology* 67: 165-183.
4. Freeman RFH, Llewellyn J. 1958. An adult digenetic trematode from an invertebrate host: *Proctoeces subtenuis* (Linton) from the lamellibranch *Scrobicularia plana* (Da Costa). *Journal of the Marine Biological Association of the United Kingdom* 37(2): 435-458.
5. Guilford HG. 1961. Gametogenesis, egg-capsule formation, and early miracidial development in the digenetic trematode *Halipegus eccentricus* Thomas. *The Journal of Parasitology* 47(5): 757-764.
6. Hanumantha Rao K. 1963. The structure of Mehlis's gland complex and histochemistry of *Artyjechinostomum mehari* (Faruqui) (Trematoda: Echinostomatidae) from a girl at Andhra Pradesh, India. *Parasitology* 53: 1-5.
7. Madhavi R, Rao KH. 1970. *Orchispirium heterovitellatum* gen. et. sp.n. from ray fish *Dasyatis imbricatus* (Day) from Bay of Bengal. *Jr. Parasitology* 56: 41-43.
8. Madhavi R. 1966. Egg-shell in Paramphistomatidae (Trematoda: Digenea). *Experientia* (Basel): 22: 93-94.
9. Madhavi R. 1968. *Dvplodiscus mehrai*: Chemical nature of egg-shell. *Exp. Parasitology* 23: 392-397.
10. Ramalingam K. 1973. Chemical nature of the egg shell in helminths. II. Mode of stabilization of egg shells of monogenetic trematodes. *Exp. Parasitology* 34(1): 115-122.
11. Smyth JD. 1954. A technique for the histochemical demonstration of polyphenol oxidase and its application to egg-shell formation in helminths and byssus formation in Mytilus. *Journal of Cell Science* 3(30): 139-152.
12. Smyth JD, Clegg JA. 1959. Egg-shell formation in trematodes and cestodes. *Exp. Parasitology* 8: 286-323.
13. Srivastava GJ. 2002. *Fishes of U.P. & Bihar*. 9th Edition. Vishwavidyalaya Prakashan, Varanasi (India). pp 78.
14. Srivastava M, Gupta SP. 1976. Egg-shell formation in *Isoparorchis hypselobagri*. *Z. Parasitenkd* 49(1): 93-96.
15. Stephenson W. 1947. Physiological and histochemical observations on the adult liver fluke, *Fasciola hepatica* L. III. Egg-shell formation. *Parasitology* 38: 128-139.