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Light microscopic study on the structure of egg shell of *Haemonchus contortus* (Nematoda)

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Abstract
The structure of the egg shell of *Haemonchus contortus*, a gastrointestinal parasite of sheep and goat was studied with the help of histological and histochemical staining techniques. The egg shell of *H. contortus* is a transparent, three- layered structure. The three layers being, an indiscernible vitelline layer, a chitinous layer and an outer uterine layer. The outer uterine layer is lipo-proteinaceous in nature. The chitinous layer consists of carbohydrates, glycogen and acid mucopolysaccarides. The vitelline layer is intensely rich in lipids.

Keywords: Egg shell, Nematoda, *Haemonchus contortus*, uterine layer, vitelline membrane

1. Introduction
The structure and events involved in the formation of egg shell in a variety of nematodes have been discussed by various workers [1, 2, 3, 4, 5]. The studies revealed that generally the egg shell was found to be composed of four layers namely an inner lipd layer, a chitinous layer, a vitelline layer, and an outer protein coat or uterine layer secreted by the cells of the uterine wall but a fifth layer was also reported in some species of nematodes. The egg shell comprising of four to five layers was reported in Ascarids [6, 7,8]. In *Ascaris*, a fifth layer of egg shell consisting of gelly like substance and forming a common covering around the eggs was seen [9, 10].
Two types of inclusions namely hyaline spheres and refringent bodies of lipoidal nature were reported in the mature oocytes [11, 12 13]. The hyaline spheres contribute to the material of the hard shell and the refringent bodies in the formation of innermost layer of egg shell [14]. The formation of first layer of the primary envelope was seen to become evident as a result of shrinkage of oocyte cytoplasm rather than the lifting of the ovum membrane [15]. The chitinous layer was derived endogenously from the glycogen accumulated in large quantities in the oocytes [6, 11].
The structure and composition of the uterine layer was studied under electron microscope by a number of workers [7, 16, 17]. This layer was observed to be secreted by the uterine wall cells which are rich in endoplasmic reticulum, glycogen reserves, protein, mitochondria and electron dense secretions. In the eggs of *Ascaris lumbricoides* and *Ascaris suum*, the uterine layer is modified to form a mammiliated layer [7]. The deposition of the uterine layer is different in different nematodes. In *Oesophagostomum columbianum* it was found to be secreted by the uterine epithelium in the form of a network of thick jelly like strands which initially form loose interconnecting envelopes around the ova [18]. As the ova rolled down the uterus, the connections were broken and the egg shells become more compact. In *Trichuris ovis* the fertilized ova pressed along the secretory uterine epithelium and its secretory granules aggregated to form a coat around the ova on the sides which came in contact. However, the polar plugs remained uncovered [19]
The present study was conducted on *Haemonchus contortus*, a trichostrongylid nematode parasitic in ruminants. Previously the process of oogenesis and egg shell formation was discussed by Singh [20]. The present study throws a light on the structure of egg shell of this highly pathogenic nematode.

2. Materials and Methods
The nematode *Haemonchus contortus* was extracted from the abomasum portion of stomach of sheep (*Ovis aries*). In order to remove debris, the nematode worms were washed in

0.85% NaCl solution. For histomorphological and histochemical studies, the worms were fixed in alcoholic Bouin's fixative and Carnoy's fixative, dehydrated in a graded series of alcohol, cleared in methyl benzoate and embedded in paraffin wax. The sections were cut at 7µm in transverse and longitudinal planes by using rotary microtome. The serial sections arranged on albuminised slides were stained. For the histochemical localization of carbohydrates, glycogen, acid mucopolysaccarides, proteins and lipids the following staining methods were used.

General carbohydrates were studied by Periodic acid Schiff's staining technique [21]. Glycogen was detected histochemically by Best's carmine staining [22] and acid mucopolysaccharides by Alcian blue staining [23]. Nucleic acids were detected by Gallocyanin chromalum [24] and Methyl green pyronin Y [25] techniques. For the localization of proteins, Mercuric bromophenol blue staining [26] and Ninhydrin Schiff's staining [27] were used. The histochemical presence of lipids was detected by Sudan black B staining [28] and Oil red O in isopropanol [29]. The slides were examined under the microscope and photo micrographed.

3. Results and Discussion

The structure and chemical composition of egg shell of *Haemonchus contortus* was studied with the help of light microscope. The fertilized egg becomes oval in shape and measure 43.3x30 µm. The egg shell of *Haemonchus* ova is a transparent, three layered structure. The layers being an indiscernible vitelline layer, a chitinous layer and an outer uterine layer. The outer uterine layer of egg shell has proteins as its main constituent as it is moderately stained with Mercuric bromophenol blue and Ninhydrin Schiff's staining. Actually it is lipo-proteinaceous in nature. A weak concentration of lipids was seen in it after staining with Sudan black B and Oil red O in isopropanol. The chitinous layer consists of carbohydrates, glycogen and acid mucopolysaccharides, being confirmed by Periodic acid Schiff, Best's carmine and alcian blua staining. The vitelline layer is intensely rich in lipids (Figures 1 to 8 and Table 1).

The process of shell formation around the mature ova starts after fertilization. A vitelline membrane is laid around the fertilized ovum, underneath which concentration of cytoplasmic granules was visible. Subsequently, this concentration gets demarcated in the form of a second chitinous layer, which is formed endogenously from the glycogen reserves of the egg cytoplasm. As the ovum roll down in the uterus it gets surrounded by granules of the uterine secretions lying in the lumen which aggregate to form loose envelope around the ovum. Subsequently this loose envelope transforms into a compact outer lipo-proteinaceous layer of the egg shell.

Among the animal-parasitic nematodes maximum work has been done on the egg shells of Ascarids but there have been few studies on the egg shells of the nematodes of Trichostrongyloidea superfamily.

Faure-Fremiet [6], Wharton [30] and Chitwood [31] and Jacobs [32] suggested that the protein coat of the egg shell was formed from the secretions of the uterine cells. Large amounts of protein was reported in the eggs of *Parascaris equorum* by Wottage [2] who suggested an endogenous origin of the outer protein coat of the egg shell. In *Aspiculuris tetraptera* Anya [11] described that the outer layer of the egg shell was lipo-proteinaceous in nature and secreted by the cells of the upper uterus, the middle layer contained some chitin in addition to proteins and the inner layer was also protein positive. Gupta and Garg [33] reported that the eggs lying in the oviduct and uterus were also highly proteinaceous. Kankal [34] accounted that the proteins formed the main structural component of the egg shell of *Tanqua anomala*. Johal and Joshi [18] reported that the protein formed the main bulk of the egg yolk in fully mature ova of *Trichuris ovis*. In *Oesophagostomum columbianum*, Johal [19] described that the chitinous layer of the egg shell was formed endogenously while the outer protein layer was secreted by the uterine epithelium.

During egg shell formation in *Haemonchus contortus* the first vitelline layer gets demarcated in the fertilized ova, this is accompanied by a simultaneous shift of glycogen granules towards the periphery which get concentrated to form the second or the chitinous layer of the egg shell, endogenously. All the previous authors working on oogenesis are in consonance about the endogenous formation of the chitinous layer. In the present study on *H. Contortus*, it was seen that an enormous quantity of secretory granules are shed into the lumen of the uterus which align around the fertilized ova in loose granular envelops. The granules subsequently condense to form regular outer wall of the egg shell which is lipo-proteinaceous in nature.

Table 1: Histochemical localization of various macromolecules in the egg shell of *Haemonchus contortus*

Name of the Macromolecule	Fixative used	Staining Technique	Egg shell layers		
			Inner Vitelline Layer	Middle Layer	Outer Uterine Layer
General Carbohydrate	Carnoy fixative	Periodic acid Schiff	---	+++	---
Glycogen	Carnoy fixative	Best's carmine	---	+++	---
Acid mucopolysaccharides	Carnoy fixative	Alcian blue	---	+++	---
Protein	Carnoy fixative	Mercuric Bromophenol Blue	---	---	++
Proteins	Carnoy fixative	Ninhydrin Schiff	---	---	++
Lipids	Carnoy fixative	Sudan Black B	+++	---	+
Lipids	Carnoy fixative	Oil Red O in Isopropanol	+++	---	+

Key to Abbreviations: ++++ very intense, +++ intense, ++ moderate, + weak, --- nil.

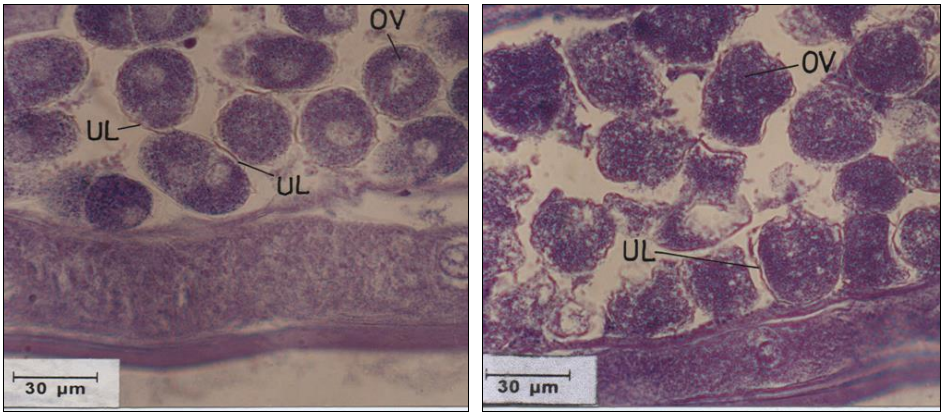

Fig 1 & 2: Transverse section of female *Haemonchus contortus* showing concentration of proteins in the outer uterine layer (UL) of ovum (OV). (Mercuric Bromophenol Blue).

Fig 3: Transverse section of female *Haemonchus contortus* showing concentration of proteins in the egg shell (SH) of ovum (OV). (Ninhydrin Schiff).

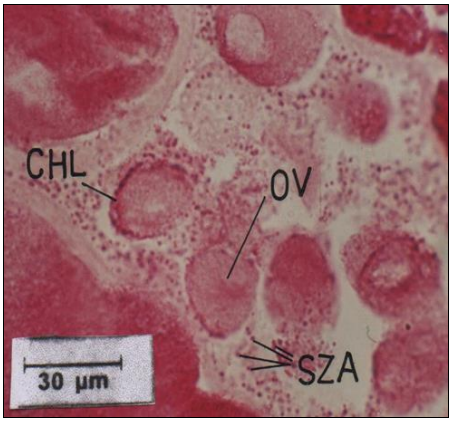

Fig 4: Transverse section of female *Haemonchus contortus* showing distribution of glycogen in the chitinous layer (CH) of ovum (OV). (Best's carmine).

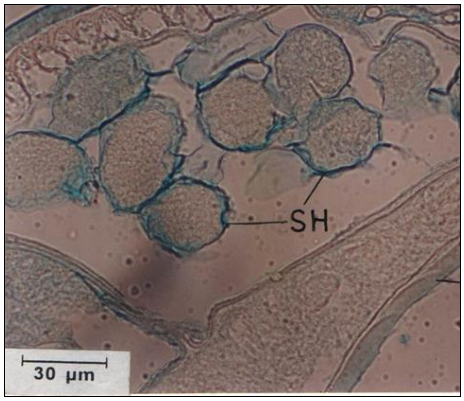

Fig 5: Transverse section of female *Haemonchus contortus* showing concentration of acid mucopolysaccharides in the egg shell (SH). (Alcian Blue).

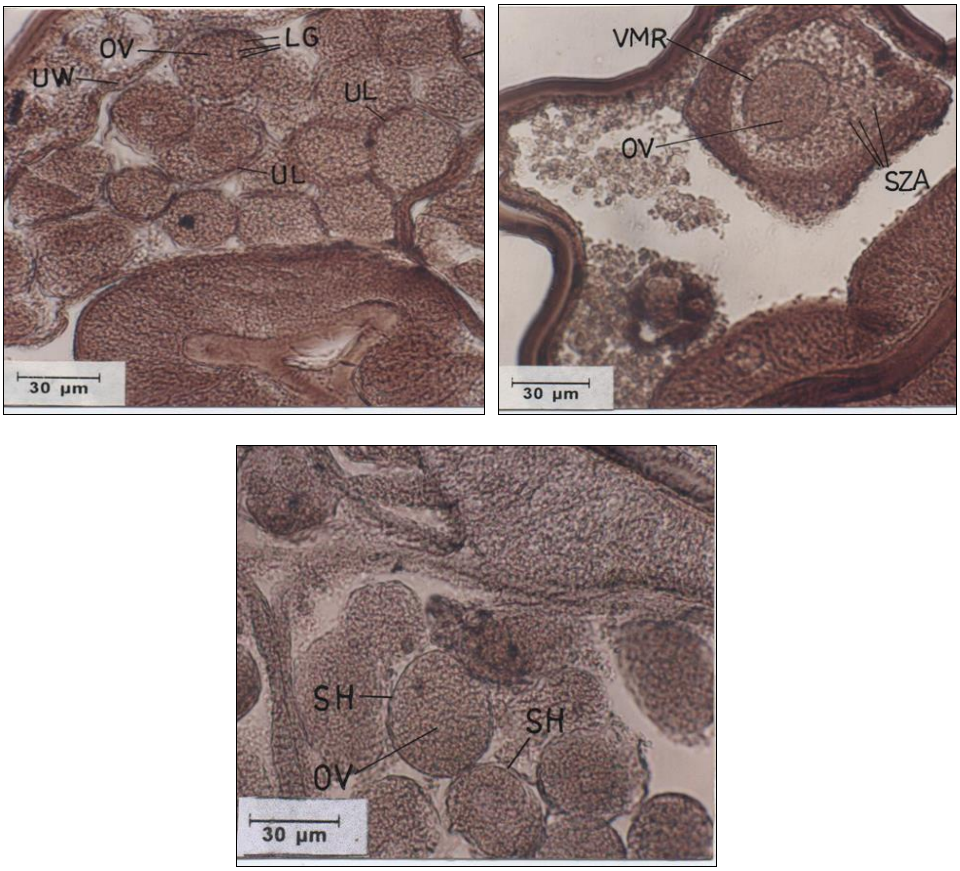

Fig 6 to 8: Transverse section of female *Haemonchus contortus* showing distribution of lipids in the egg shell (SH), lipid granules (LG), ovum (OV), spermatozoa (SZA), Uterine layer (UL), uterine wall (UW), vitelline membrane (VMR). (Oil red O in isopropanol).

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