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Study on histochemical localization and specific activity of alkaline phosphatase in *Cotylophoron cotylophorum* (Paramphistomidae: Digenea), recovered from rumen of cattle

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Abstract
In the present study the detailed histochemical localization of alkaline phosphatase at the tissue level of *Cotylophoron cotylophorum*, a stomach fluke of cattle, has been described. *Cotylophoron cotylophorum* were collected from the rumen of cattle in local abattoirs. Frozen sections were cut and histochemical localization of Alkaline phosphatase was done by using Calcium Cobalt method [17] in which alkaline phosphatase deposits appear black or brownish black. In the present study substantial amount of alkaline phosphatase was observed in the various structures of *Cotylophoron cotylophorum*. Intense reaction for alkaline phosphatase was observed in intestinal caeca, excretory vesicle, tunica of testes and ovary. Moderate reaction for alkaline phosphatase was observed in oral sucker, acetabulam, vitellaria, tegument and sub-tegument of *Cotylophoron cotylophorum*. Parenchyma showed weak reaction for alkaline phosphatase in the present study. The possible functional role of alkaline phosphatase in different tissues has been discussed.

Keywords: histochemistry, alkaline phosphatase, *Cotylophoron cotylophorum*, cattle

Introduction
Amphistome *Cotylophoron cotylophorum*, a digenetic trematode, is parasitic in the alimentary canal of many ruminants. Mature parasites are especially prevalent in the reticulum and rumen. Immature migrating parasites have been reported causing serious disease and even the death of their hosts by burying themselves in the sub mucosa of duodenum and feeding on the epithelial cells of Brunner's glands which results in anorexia, polydypsia, profuse diarrhoea, a drop in plasma protein concentration and anemia [1, 2, 28]. However, mature paramphistomes rarely produce clinical symptoms [4, 11]. The survival of the parasites is influenced by the general biotic factors associated with the micro and macro environments as well as by the intimate physiological and immunological interactions between the parasite and the host which forms the basis of host-parasite relationship. The histochemical studies help us to investigate qualitatively the biochemical pattern of different tissues in cellular architecture. Histochemical studies will increase the knowledge of worm physiology, which could further lead to deeper understanding of the well recognized host parasite interactions and such information would be valuable in designing control measures that are efficient and economical [22]. The purpose of the present study was to examine the histochemical distribution of alkaline phosphatase in *Cotylophoron cotylophorum.* Such studies could increase our knowledge of the biochemistry of the parasites, which could further lead to deeper under standing of well recognized host parasite relationship.

Material and Methods
The live amphistomes were collected from rumen of freshly slaughtered cattle in local abattoirs. Worms were carefully removed with the help of fine forceps, placed in normal saline (0.75%) and washed thoroughly. The worms were fixed in cold Neutral Formalin for 1- 4 hours in deep freezer and washed thoroughly with running water. The frozen sections 8 – 10 microns thick were cut at – 20 ^{0}C by using *Leica CM 3050 S* cryostat. Frozen sections 8 – 10 micron thick were mounted on clean slides without any adhesive, dried in air at room temperature for 1-2 hours and incubated in Incubating solution(containing substrate for alkaline phosphatase-Sodium-β-Glycerophosphate) for ½ - 4 hours at 37 ^{0}C. The sections were then washed in running water and treated with 2% Cobaltous acetate solution for 3 – 5 minutes. After this the sections were again washed in water and given the treatment of dilute yellow ammonium sulphide for 1 – 2 minutes. Again washed in water and counter stained in aqueous 1% Eosine. Finally the sections were washed in water and mounted in glycerin jelly. Control sections were incubated in a solution with out substrate.

Results
In the present study substantial amount of alkaline phosphatase was observed in the various structures of *Cotylophoron cotylophorum*. Intense reaction for alkaline phosphatase was observed in intestinal caeca, excretory vesicle, tunica of testes and ovary. Moderate reaction for alkaline phosphatase was observed in oral sucker, acetabulam, vitellaria, tegument and sub-tegument of *Cotylophoron cotylophorum*. Parenchyma showed weak reaction for alkaline phosphatase in the present study. The statement of staining reaction for alkaline phosphatase in different tissues/organs of *Cotylophoron cotylophorum* is presented in Table 1. Fig.1–6 show the histochemical distribution of alkaline phosphatase in

various tissues of *Cotylophoron cotylophorum*.

Table 1: Intensity of Alkaline phosphatase staining reaction in the tissues of *Cotylophoron cotylophorum*

Tissue/ Organ	Alkaline phosphatase Activity
Tegument	++
Tegumental muscles	++
Pharynx	++
Oral sucker	++
Acetabulam	++
Intestinal caeca	+++
Tunica of testes	++
Tunica of ovary	++
Parenchyma	+
Vitellaria	++
Excretory vesicles	+++

+ Weak activity, ++ Moderate activity, +++ Intense activity

Fig 1

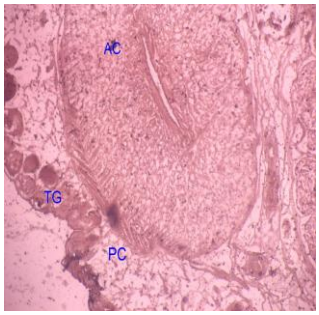

Fig 2

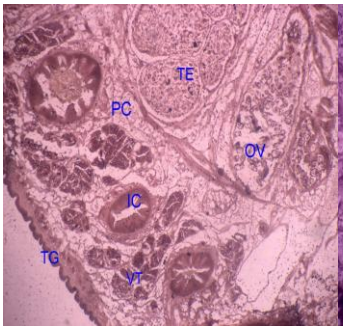

Fig 3

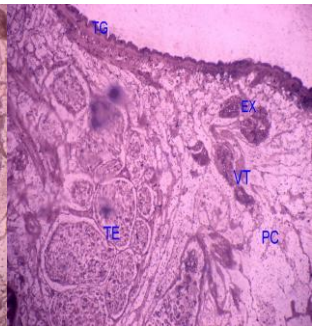

Fig 4

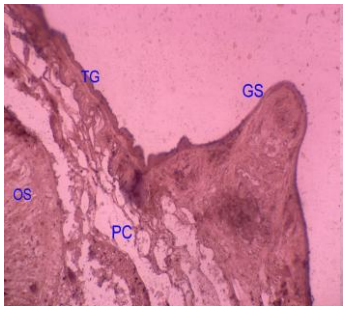

Fig 5

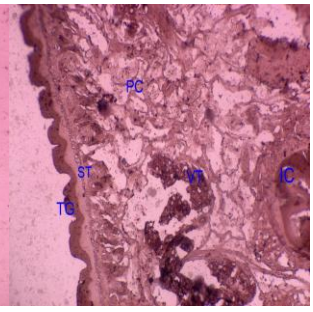

Fig 6

Fig 1: LS through OS (oral sucker) and PH (pharynx).x200. **Fig 2:** LS through AC (acetabulum).x200. **Fig 3&4:** LS through TE (testes), OV (ovary), TG (tegument), EX (excretory duct), VT (vitellaria) and PC(parenchyma).x200. **Fig 5:** LS through GS (genital sucker) x200. **Fig 6:** LS through IC (intestinal caeca), ST (subtegument) and TG (tegument).x200.

Discussion

It is commonly known that alkaline phosphatase is the enzyme functionally associated with membrane transport and its role mostly lies in participation in active transport of nutrients and metabolites through the cellular membranes [29]. Diverse and multilateral functions are attributed to alkaline phosphatase. In particular, it is assumed that alkaline phosphatase takes part in the regulation of NADP levels, in proliferation and differentiation of cells and also in the regulation of cell membrane dimensions [24, 12]. Similarly diverse functions are attributed to alkaline phosphatase in parasites. The level of alkaline phosphatase is associated with the synthesis of cytoplasmic proteins and with the cell growth [29]. On the other hand [5, 3, 13, 19], discovered an antigenic character of this enzyme in *Schistosoma mansoni*. Alkaline phosphatase is also a sensitive indicator of viability of the developing embryos of *Schistosoma mansoni*, and lack of alkaline phosphatase activity in the egg is a first sign of their death [6].

In the current study, substantial amount of alkaline phosphatase was found in the studied structures of *Cotylophoron cotylophorum* which is probably associated with intense transport of carbohydrates constituting the major source of energy for the parasite.

Moderate reaction for alkaline phosphatase was observed in the suckers of the amphistome during this study. The reaction products were localized all along the tegumental surfaces of suckers having direct contact with the host tissue and may have glandular activity. The distribution is more or less similar to that reported by [10, 23] in *Ceylonocotyle scoliocoelium*. The presence of phosphatases in the suckers indicates that they may be involved in carbohydrate metabolism and absorption of nutrients as well as in dissolving host tissue at the host – parasite interface for extracorporeal digestion.

Moderate to intense reaction was observed in the tegument and sub tegument for alkaline phosphatase in *Cotylophoron cotylophorum* and the results are in agreement with those of [23, 15, 27, 31]. Moderate to weak activity for alkaline phosphatase in the tegument of Juvenile paramphistomes is also reported [15]. Similar results for alkaline phosphatase were reported by [27] in the tegument of *Orthocoelium scoliocoelium* and *Paramphistomum cervi*. It is suggested that phosphatases are related to transport mechanism, the identical distribution of enzymes in the tegument and gut suggests that nutrients are absorbed through both gut and tegument. The presence of moderate amount of phosphates in the tegumental syncytium suggests that at least part of these paramphistomes nutritive requirement is met by transtegumental absorption [15].

Present study has revealed the presence of alkaline phosphatase in high concentration in the gut caeca. These findings are in agreement with those of [16, 10, 23, 15] in *Cotylophoron cotylophorum*; *Gastrothylax explanatum* and *Gastrothylax crumenifer*; *Ceylonocotyle scoliocoelium* and juvenile paramphistomes. Alkaline phosphatase in the gut indicates its involvement in the digestive physiology of parasite probably by the process of dephosphorylation and phosphorylation. Moderate to high amount of alkaline phosphatase was demonstrated in organs of reproductive system. Intense reaction was observed in the tunica of testes and ovary, while moderate to weak reaction in the testes and

ovary. These findings are in agreement with those reported in *Aspidogaster conchicola* (Trematoda) [31]; in *Fasciola hepatica*, *Schistosoma mansoni* and many other trematodes [18, 26, 30]. In trematodes, organs which are metabolically highly active such as gonads, must rely on surrounding parenchyma as pool of nutrients, the presence of alkaline phosphatase at these sites facilitates the transport of raw materials in to the reproductive organs.

In the present endeavour vitellaria revealed moderate activity for alkaline phosphatase in the amphistome under study. The results are in agreement with most of the workers in various trematodes [7, 8, 9, 20, 21, 26]. Vitelline glands are important in egg shell formation and the raw materials such as sugars, fatty acids and amino acids needed for this purpose are transported by the activity of alkaline phosphatase. The present study has also revealed the presence of alkaline phosphatase in parenchyma and excretory ducts. Weak reaction was observed in parenchyma. Similar results were reported by [14, 23, 18, 10] in *Paramphistomum epiclitum* and *Fischoederius elongatus*; *Ceylonocotyle scoliocoelium*; *Fasciola gigantica*; *Gastrothylax crumenifer*. Just like in other organs, the presence of alkaline phosphatase in the parenchymal tissue indicates its involvement in the phosphorylation of absorbed substances which might ensure there retention. Presence of alkaline phosphatase in the excretory vesicles suggests that the enzyme facilitates the absorption and dislodgement of metabolic end products.

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