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Ultrastructural observations of rumen immature and mature *Paramphistomum cervi* (Trematoda: Digenea) in domestic buffalo of Udaipur district, Rajasthan

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Abstract
Worldwide spread, especially in Asia, group Digenea is most diverse group of trematoda among others groups, capable to infest most of classes of vertebrates with support of invertebrate group (intermediate host). Digenean flukes can rigorously infect domestic ruminants as well as human, and become responsible for amphistomiasis disease. *Paramphistomum cervi* is digenean parasitic worm belonging to the class Trematoda, it is a tiny worm mostly parasitising domestic ruminants; buffalo, cow, sheep & goat and it cause severe diseases called paramphistomiasis. *Paramphistomum cervi* are commonly occurring species, aggressively spread in domestic buffalo of southern Rajasthan.
Trematode includes worms which are endo-parasites having different shape, size, number, size of suckers, cuticle, oral organ, oesophagus, intestinal caeca, excretory vesicle, genital bulb, male and female reproductive organs. SEM photomicrograph revealed that the body of mature *Paramphistomum cervi* in conical shape and the anterior end narrow and the posterior being broad, and pink in colour. The tegumental surface is highly ridged with transverse folds discontinuous with grooves and is spineless, which is uncharacteristic of trematodes. The genital pore is situated at the anterior third of the body. There are two types of bulbous shaped sensory papillae on the surface, situated at the base with nipple-like tips.

Keywords: *paramphistomum cervi*, ultrastructure, SEM study, domestic buffalo, paramphistomiasis, immature and mature worm

Introduction
Worldwide spread, especially in Asia, group Digenea is most diverse group of trematoda among other groups, capable to infest most of classes of vertebrates with support of invertebrate group (intermediate host). Digenean flukes can rigorously infect domestic ruminants as well as human, and become responsible for amphistomiasis disease. This pathogenic disease can observe in both wild and domesticated animals' plays important role in economic loss to poor cattle farmer of developing countries as well as threat to human health (Rajakaruna and Warkulasooriya, 2011; Crotti, 2013 and Kanwal *et al*., 2014) [41, 13, 25].

Paramphistomum cervi is digenean parasitic worm belonging to the class Trematoda, it is a tiny worm mostly parasitising domestic ruminants; buffalo, cow, sheep and goat and it cause severe diseases called paramphistomiasis. This parasitic disease is responsible for significant losses through morbidity and mortality in domestic animals in entire world (Njoku and Nwoko, 2009; Raza *et al*., 2009; Ozdal *et al*., 2010; Melaku and Addis, 2012 and Iqbal *et al*., 2013) [32, 42, 35, 30, 31]. Parasitism is the major cause delaying the development of domestic animals population in throughout the world (Farooq *et al*., 2012; Elele *et al*., 2013 and Swarnakar *et al*., 2014a & b) [17, 16, 43].

Paramphistomum cervi are commonly occurring species, aggressively spread in domestic buffalo of southern Rajasthan. Trematode includes worms which are endo-parasites having different shape, size, number, size of suckers, cuticle, oral organ, oesophagus, intestinal caeca, excretory vesicle, genital bulb, male and female reproductive organs. Ultra-micrographs give fundamental sketch of the structure of paramphistomes species comprises small, curved, conical or pyriform body with well-developed oral organ, broad fleshy body with posterior acetabulum, non-spinous cuticle, terminal mouth without oral pouches, excretory pore is dorsal and Laurer's canal opening posterior to excretory vesicle, distinct genital bulb (Tandon and Maitra, 1981 and 1982; Xylander & Poddubnaya, 2009; Panyarachun *et al*., 2010; Ashour *et al.*, 2011; Anuracpreeda *et al.*, 2012; Panyarachun *et al.*, 2012; Sotillo *et al.*, 2012 and Panyarachun *et al.*, 2013; Swarnakar *et al.*, 2014; Swarnakar & Kumawat, 2016 and Swarnakar & Damor, 2016) [58, 59, 36, 37, 38, 5, 48, 52, 51].

Electron microscopy has established their uses in the field of various types of parasites study. It accumulates lot of information about parasite which is useful for different aspects of biology and pharmacology. It also deals with study of development stages of trematode parasite that increase ultrastructure knowledge to biologist for future aspects (Jamjoom and Shalaby, 2006) [24]. Present observations are to conclude the structure differences between immature and mature *Paramphistomum cervi* by SEM, which will be helpful in species identification and prepare medicine for their diagnosis.

Materials and methods
For the identification of species *Paramphistomum cervi* in rumen samples
Immature and mature *Paramphistomum cervi* were collected from infected parts of rumen of domestic buffalo (*Bubalus bubalis*) at local zoo abattoir and various slaughtered houses

of Udaipur. The infected part of rumen was brought periodically to the laboratory and these worms washed several times in the tap water and they were transferred into 0.9% physiological saline, fixed in hot AFA (Alcohol 85 ml, formalin 10 ml and acetic acid 5ml.) at 80 to 85° C for few second then pressed between two slides (to make them flat) left in cold AFA, bleached in chlorinated alcohol for twelve hours. Bleached worm washed in 70% alcohol, stained with alcoholic borax carmine for 5 min, dehydrated in alcoholic series and cleared in clove oil for twelve hours. Cleared *Paramphistomum cervi* mounted in DPX on glass slides and examined under light microscope. The whole mounts of parasites used for identification of the worms, on the basis of their morphological character as detailed by Dutt, (1980) [14].

For the electron microscopy of *Paramphistomum cervi*

Saline washed worms were fixed 12 hrs at 4ºC in 3.5% glutaraldehyde in 0.2M sodium cacodylate buffer at pH 7.2 fixative (Bancroft and Stevens, 1977) [7]. Some of the fixed parasites sent to the Regional Electron Microscopy Facilities, AIIMS, New Delhi. Where, the worms were post fixed at 4ºC for 1 hour in 1% osmium tetraoxide prepared in 0.1M phosphate buffer at pH 7.2 and dehydrated in acetone ascending series and then transferred to 100% acetone for one hrs. and dried. Later on, the worms glued on metal stubs, coated with gold in vacuum and examined by using ZEISS Scanning Electron Microscope (SEM).

Result

Rumen removed immature and mature *P. cervi* studied by SEM, SEM photomicrographs are exposing the body of *Paramphistomum cervi* in conical shape and the anterior end narrow and the posterior being broad. The worm measures about 5–13 mm in length and 2–4 mm in width across the midsection. It has two suckers, an anterior sucker (AS) or oral sucker and a larger posterior sucker (PS) or acetabulum. The tegumental surface of *P. cervi* is highly ridged with irregular transverse tegumental folds (TF) with grooves and spineless. The surface of the fold is composed of micro-folds separated by microgrooves at higher magnification. The major folds were located in the areas of the anterior sucker, genital pore, and posterior sucker (Plate 1). In adult worms, the entire tegument covered with papillaes; showed 2 kinds of papillaes, but in immature worms showed far simpler external structure; they lacked fully developed and functional structures of papillaes only swelling-like structure appeared.

Immature worms demonstrated a far simpler external structure than mature worm. In Immature worm, tegumental surface is highly corrugated with many transverse tegumental folds (TF) discontinuous with multiple grooves (G) than mature, because of under developing reproductive structure, and is spineless (Plate 6). The entire tegument of *P. cervi* covered with different sized papillaes. In addition; both suckers, which are modification of tegument, of immature and mature worm appeared approximately similar with numerous papillaes, less developed visible papillaes observed in immature, because sucker helps to worm in adhesion and consume nutrients vigorously to grow in a host body (Plate 2 and 3).

The genital pore (GP) is located at the anterior third of the body of worm. Genital pore contrastingly visible in Plate-4 of both animals, thorough study revealed that genital pore of immature having less number of papillaes and as well as found inactive. In Plate-5 and figure 1 revealing genital pore with ejaculatory duct in immature that state presenting the protoandry condition; sometimes male organs develops before female but exhibits immature position. Same plate but figure 4 displaying mature genital pore with ejaculatory duct. There are two types of bulbous shaped sensory papillae (SP) observed on the surface, situated at the base with nipple-like tips around the genital pore. Each dome-shaped papilla is elevated above the surface. Anterior end found large size papillaes (P) as compared to posterior end in both worm but in immature worm; outward surface sprouting dot-like appearance of developing papillaes (DP) are noticed, not completely developed and functional. Clusters of papillae on the ventral surface and around the anterior suckers are larger in size and number while dorsal side of the body demonstrate similar surface features, but papillae appear fewer and smaller, and mountain like resemblance was seen on surface (Plate 1,2,3,4 and 5).

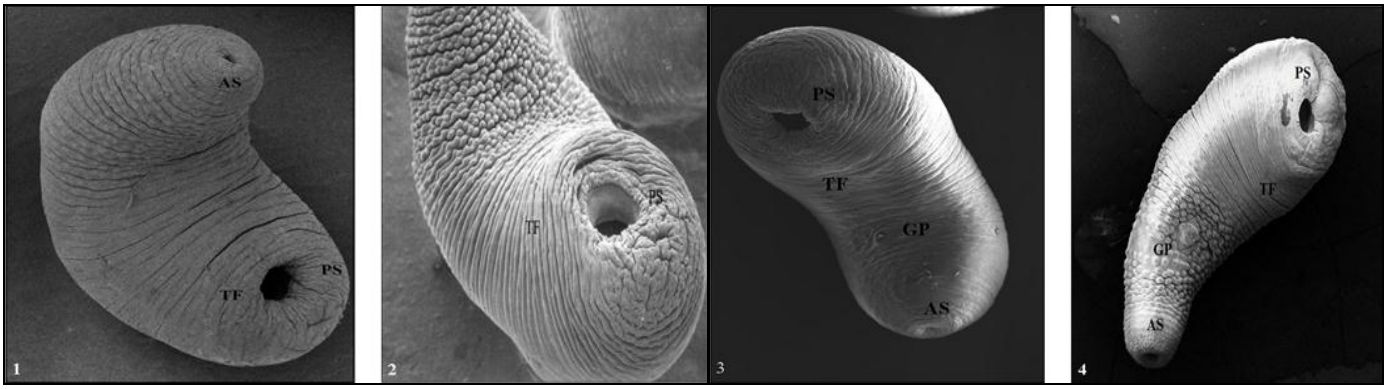

Fig 1, 2, 3, 4: SEM full photomicrograph of immature (1&3) and matur (2&4) P. cervi showing cokparative structures of posterior sucker (PS), Anterior sucker (AS), Genital pore (GP) and Tegumental folds (TF).

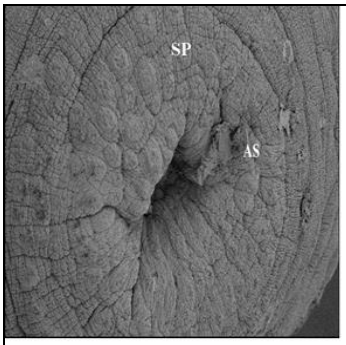

Fig 5: Anterior sucker (AS) of Immature *P. cervi* with sensory papillaes (SP) in developing condition.

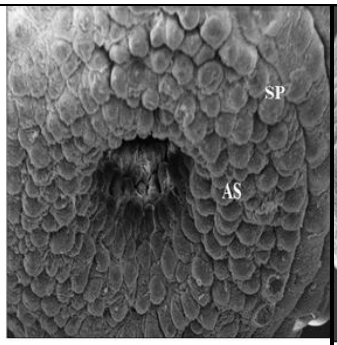

Fig 6: Anterior sucker (AS) of Mature *P. cervi* with sensory papillaes (SP) in developed condition.

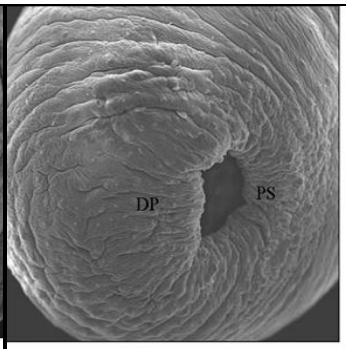

Fig 7: Posterior sucker (PS) of Immature *P. cervi* showing developing Papillaes (DP).

Fig 8: Posterior sucker (PS) of mature *P. cervi* showing developed Papillaes (P).

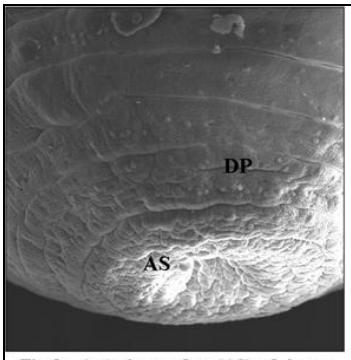

Fig 9: Anterior sucker (AS) of immature *P. cervi* with Developing papillae (DP).

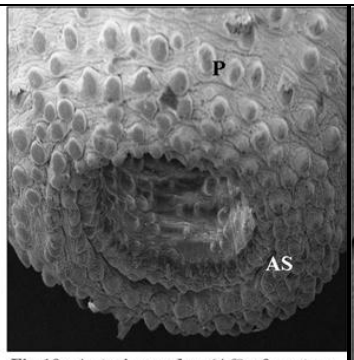

Fig 10: Anterior sucker (AS) of mature *P. cervi* with fully Developed papillae (P).

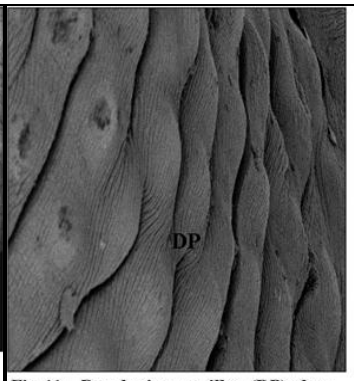

Fig 11: Developing papillae (DP) showing in immature *P. cervi*.

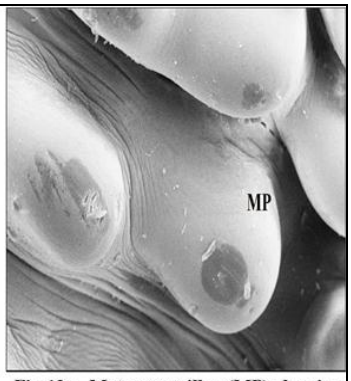

Fig 12 : Mature papillae (MP) showing in mature *P. cervi*.

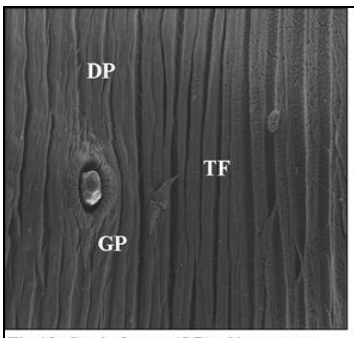

Fig 13: Genital pore (GP) of immature *Paramhistomum cervi* showing developing papillae (DP) and Tegumental fold (TF).

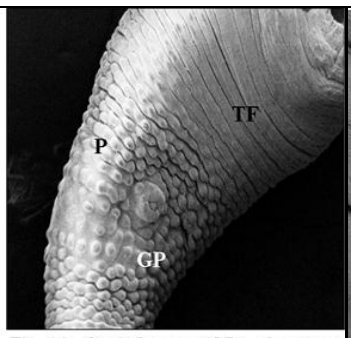

Fig 14: Genital pore (GP) of mature *Paramhistomum cervi* showing developed papillae (P) and Tegumental fold (TF).

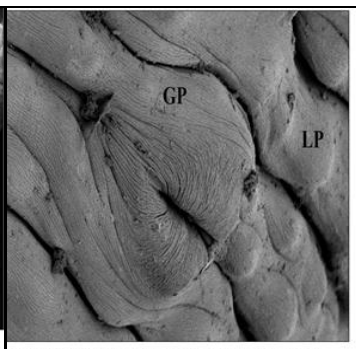

Fig 15: Genital pore (GP) of immature *Paramhistomum cervi* around large papillae (LP).

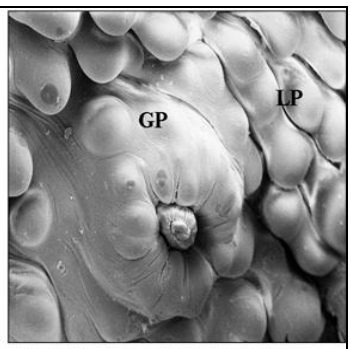

Fig 16: Genital pore (GP) of mature *Paramhistomum cervi* around large papillae (LP).

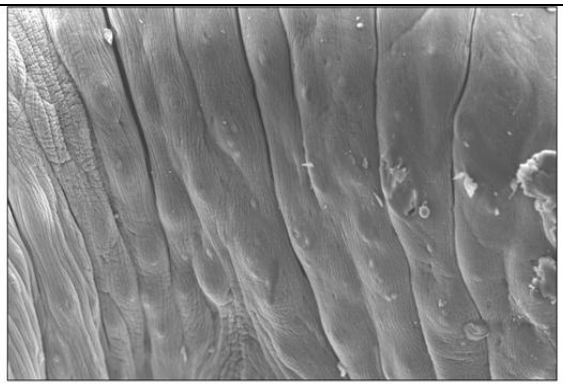

Fig. 17: Underdeveloped papillaes observed in immature worm.

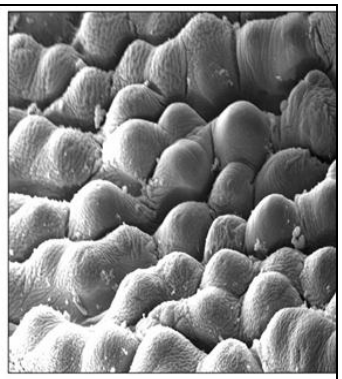

Fig. 18 & 19: Fully mature and developed surface papillaes found in mature .

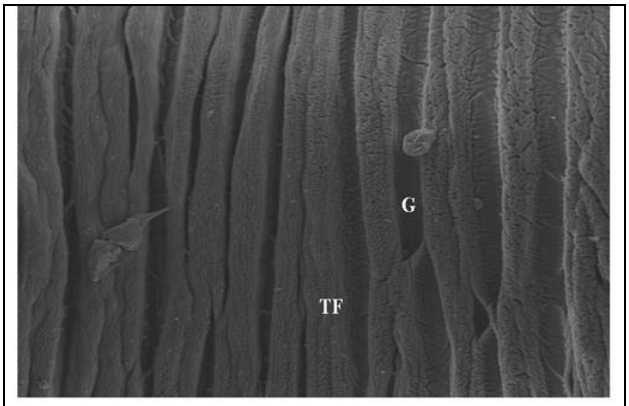

Fig. 20: Highly corrugated tegumental surface of immature worm

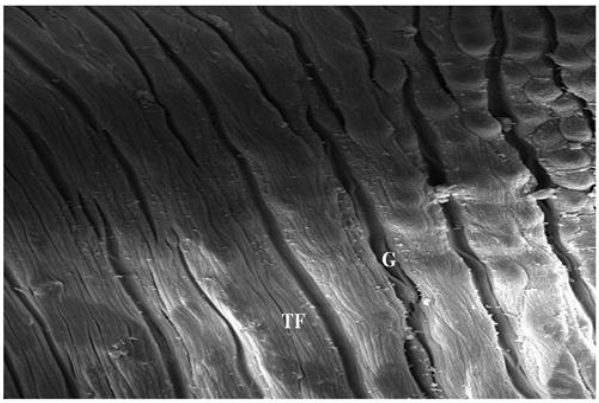

Fig. 21: Tegumental surface of mature worm with tegumental folds (TF) and having multiple grooves (G).

Discussion

Trematoda class shows various distinguish features of belonged animal that helps in isolation of included members from others class members. These structures play major role in continued existence of the class animals; body covered by integument with often bears spines papillae or tubercles, well developed reproductive system, having alimentary system consists oral and posterior suckers with absence of anus except Fasciola species and major feature of this class animals having two hosts; primary host (Sexual reproduction) and intermediate host (Asexual reproduction).

Ultrastructure is the nano or fine structure study of a biological sample such as a cell, tissue and organ, at very higher magnification by electron microscopy. These studies help to provide morphological knowledge and direction to prepare the medicine for their treatment. Surface features by scanning electron microscope were exhibited in the different digenean: *Astiotrema impletum, Cotylophoron cotylophorum, Explanatum explanatum,, Fasciola gigantica, Gorgodorina vitelliloba, Orthocoelium scoliocoelium, Phyllodistomum umblae, Schistosoma haematobium*, *Schistosoma mansoni* and *S. japonicum (*Bennet, 1975b; Abdul-Salam and Sreelatha, 2000; Hamada, 2002; Jamjoom and Shalaby, 2006; Ichikawa *et al*., 2012; Swarnakar *et al*., 2014b; Swarnakar & Kumawat, 2016 and Swarnakar & Damor, 2016) [1, 9, 52, 51, 19, 24].

Simon-Martin and Rojo-Vazquez, (1984) [46] explained differentiation between mature and immature digenean worm Sanguinicola species in their study that the mature worm, the entire tegument covered with microvilli; showed various kinds of sensory organs and the immature worms showed a far simpler external structure; they lacked microvilli but did show orifices which could be sensory organelles.

The tegument plays important role in protection, absorption, excretion, secretion, transportation and osmoregulation. It is the surface which is in direct contact with host's tissue along with the body fluids (Otubanjo, 1985; Sharma and Hanna, 1988; Brennen *et al*., 1991; Irwin *et al.,* 1991; Shuhua *et al.* 2000; Srimuzipo *et al.* 2000, Ashour *et al*., 2001; Panyarachun *et al*., 2010; Shaheen and Eman, 2012; Saowakon *et al*., 2013 and Hadied *et al*., 2015) [36, 34, 45, 23, 49, 18]. In addition; tegument is highly folded, folding surface encircle the whole body, and invaginated into numerous ridges, pits and spines**,** which help to increases the surface area of the tegument for the absorption and exchanging of needy molecules from host body, as well as for attachment and these are more numerous near the anterior end and fewer posteriorly (Tandon and Maitra, 1982; Tondon and Maitra, 1987; Sethukarasi & Veerakumari 2007 and Rajesh *et al*., 2017) [58, 57, 56, 43]. Suckers approximately show similar function as tegument except adhesion in host body because sucker is modification of tegument. Sobhon *et al*., (1998) [47], Brennan *et al*., (1991) [10], Staudt *et al*., (1992) [50], Swarnakar *et al*., (2014) and Swarnakar & Damor (2016) [51] studied the tegument of different trematodes respectively; *Fasciola gigantica*, *Gastrodiscoides hominis, Schistosoma mansoni, Orthocoelium scoliocoelium* and *Fasciola gigantica*, tegument of the trematodes is folded into concentrically arranged furrow and ridges bearing numerous tightly packed tubercles extends into the oral cavity and ciliated & non-ciliated papillaes are found around the oral openings, that features helps the parasite to maintain its homeostasis, and avoid the unfriendly environment in a host body, including the host's immune attacks. Not only in adult fluke but only cercariae of trematode having tegument with small microvilli shows function in nutrient absorption (Koiea 1987; Lee *et al*., 1987) [26, 28]. These mentioned features about tegument shows that having a tegument structure are most important and necessary tissue of trematode parasite for survival in animal host body.

Bakke, (1975) [6], Kotrla & Chroust, (1978) [27], Otto Sey, (1985) [33], Imkongwapang *et al*., (1999) [22] and Miranda & Costa, (1999) [31] stated in their papers, three types of sensory structures have been distinguished on body tegument of different species of amphistomes from different vertebrate hosts (in Fishes, amphibians, reptiles and mammals). Present observations favour that trematode *P. cervi* bearing only 2 types of papillaes unlike above observations. Thus, types of papillaes may vary animal to animal. Papillaes greatly increase the surface area of tegument which helps in consume nutrients and respiration. The tegumental papillae, combined with other characters, might be useful set of traits in amphistome diagnosis (Bennet, 1975a) [8].

General features of trematode are conical or oval to pyriform in shape, thick at the mid-region from anterior of posterior and ventral highly corrugation and invaginations than those of the dorsal surface of the body with grooves and with or without spines. Generally, trematode has two types of sensory papillae on the surface: type1 is bulbous in shape and nipple-like tips and also type 2 is a similar shape but has short cilia on tips

sometimes without cilia. These sensory papillae occur in large clusters. Clusters of papillae on the ventral surface and around the anterior suckers look more numerous and large in size. The dorsal side of the exhibit similar surface features, but papillae appear less in number and size. Dome-shaped or bulbous papillae with smooth apical surface are found scattered on the ventral surface but were concentrated around the oral and ventral suckers and irregular borders were randomly scattered on the anterior and in concentric rows around the genital pore opening (Ahmed *et al*., 1987; Eduardo and Henry 2007; Seck and Ba 2007; Conn *et al*., 2008; Lim *et al*., 2008; Xylander and Poddubnaya, 2009; Ashour *et al.*, 2011; Anuracpreeda *et al*., 2012; Chae *et al*., 2012; Panyarachun *et al*., 2012; Sotillo *et al*., 2012; Panyarachun *et al*., 2013; Radwan *et al*., 2014; Swarnakar *et al*., 2014; Anuracpreeda *et al*., 2016; Shalaby *et al*., 2016 and Swarnakar & Kumawat, 2016) [52, 44, 15, 12, 59, 5, 11, 37, 38, 39].

Present study has similar observations with Panyarachun *et al*., (2010) [36] stated that adult *Paramphistomum cervi* are pear-shaped, slightly bulging inward ventrally and bulging outward dorsally. The tegumental surface in all part of the body appears highly corrugated with transverse folds alternating with grooves and is spineless. The surface of the fold is composed of micro-folds separated by microgrooves at higher magnification. Ventral surface are more corrugated and invaginated than other surface of the body. Anterior and posterior suckers both have thick covering with transverse folds without spine.

Such studies on the other ruminant parasites are necessary because many of them are responsible to cause paramphistomiasis. In this study, we attempted to ultrastructural characteristics of immature and mature *Paramphistomum cervi* that could increases the knowledge and understanding of rumen fluke surface topography as well as suggests that synthetic drugs and herbal treatments more effective for immature than mature worm because immature condition exhibits, worms are reproductively inactive as a result; they are failed to increase their population, for that reason this is the best timing for curing the domestic buffalo without any pain and can cut off all the symptoms. It might be become severe in future if treatments processes not receive on time but removal of worm in earlier stage (Immature) supposing restore condition of domestic buffalo speedy before extreme damage means mortality not occurs, whereas, mature worm may harm severely and may cause mortality.

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