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PATHOLOGICAL STUDY OF INTESTINE IN THE INDIAN BULL FROG, *Hoplobatrachus tigerinus* DAUDIN PARASITIZED WITH *Tremiorchis ranarum* (DIGENEA: PLAGIORCHIIDAE) FROM YSR DISTRICT, ANDHRA PRADESH, INDIA

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration between both authors. Author APV designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author HM managed the literature searches and analyses of the study. Both the authors read and approved the final manuscript.

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ABSTRACT

The Indian bull frog, *Hoplobatrachus tigerinus* Daudin was found to be parasitized with the digenean, *Tremiorchis ranarum* Mehra et Negi. During an investigation of helminth parasites of amphibians from several localities of YSR district, Andhra Pradesh, this parasite was recovered from the intestine of the host. The morphological and diagnostic characteristics of *Tremiorchis ranarum* was studied by means of light microscopy, scanning electron microscopy along with its pathological effects on the host intestinal tissues to assess the extent of damage caused by them. Histopathogical effects include destruction of intestinal villi, inflammatory fibrosis, inflammation of villi at the site of attachment of parasite, hyperplasia and metaplasia, vacuolation of sub-mucosal cells and degeneration of intestinal layers due to proliferative changes. The pathological effects also include an increase in the thickness and the damage to mucosa.

Keywords: Histopathology; *Hoplobatrachus tigerinus*; *Tremiorchis ranarum*; YSR District; metaplasia; hyperplasia.

1. INTRODUCTION

Amphibians serve as a crucial part of their ecosystems as they can serve as effectual biological indicators of environmental health De Maynadier and Hunter, [1]. The parasitic infections in wild populations of amphibians are exceptionally common due to their dual mode of life (aquatic and terrestrial habitat) where the ecological requirements for intermediate hosts and parasitic transmission are easily met

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Maisam et al., [2]. But unfortunately, these amphibian populations are drastically declining at alarming rates due to anthropogenic activities and infectious diseases caused by viruses, bacteria and parasites Vie et al., [3]. The pathogenicity of preferred parasites can be examined using histopathology Williams, [4], Feist and Longshaw, [5]. Frog diseases and histopathology are progressively being used as markers of environmental stress as they present a distinct biological end point of histological exposure. This mechanism can be absolutely considered as an imperative tool in determining the effect of parasites on frog tissues. Intestinal parasites of vertebrates can induce inflammation of the host digestive tract, resulting in altered gastrointestinal function, namely enhanced secretion and propulsive motility of the gut Palmer and Meerveld, [6]. Also, on the basis of attachment mechanism, these parasites can fatally disturb the veracity of the mucosal gut layer by inducing lesions ranging from shallow erosions to deep ulcerations with hemorrhages and perforation of the gut wall, further resulting in peritonitis and septicemia with the lethal outcome, which is rarely noticed in the wild, except in cases of mass mortalities. The precise information of histology gives a fringe to distinguish the changes induced by the parasites in the infected tissue from the uninfected (control) tissues. Bose and Sinha [7], Bilqees and Fatima [8], Bilqees and Parveen [9], Campos et al., [10], Benarjee and Reddy [11,12], Nero et al., [13], Camargo and Martinez [14], Vankara and Chikkam [15,16], Dap [17], Abalaka et al*.,* [18], Raisy and Ansari [19], Mohammadi et al.,[20], Padmini and Kumar [21], Zenon and Magdalena [22], Fartade and Fartade [23], Ozdemir et al., [24], Palaq et al*.,* [25,26] are few authors who contributed on the pathological and histochemical changes induced by the parasites on the different tissues of various hosts especially on the fishes but there are only a handful of histopathological studies on the frogs by Martin and Conn [27], Nieto et al., [28], Miller et al., [29], Al-Attar [30], Maisam et al., [2]. For the present study, the Indian bull frog namely, *Hoplobatrachus tigerinus* was found to be infected with the digenean parasites. Helminth parasites, especially endoparasites infect the regions of stomach, intestine and rectum. Intestine is considered to be the most favorable and preferred site offering a prospective niche and a wealth of constant supply of nutrients to digeneans as they are parasites and have incomplete digestive system. Digeneans usually adhere to the intestinal wall of the hosts with the aid of oral and ventral suckers causing mechanical damage and absorbing host's nutrients and host's digestive enzymes. Very few reports on the histopathology of anurans infected by the digenetic trematodes were noted Molnar, [31]; Reddy et al., [32]; Patil and Chaudhari, [33]; Reddy and Benarjee, [34]. *Tremiorchis ranarum,* a digenean parasite inhabiting the intestine of the Indian bull frog, *Hoplobatrachus tigerinus* is one of the frequently occurring species occupying the position of satellite species (11.3%). In the present study, an attempt was made to observe the morphological features of *T. ranarum* and its pathological effects at the site of attachment in the intestine by comparing with the control (uninfected) tissues.

2. MATERIALS AND METHODS

2.1 Sample Collection and Examination

Adult frogs, *Hoplobatrachus tigerinus* (n =300) (Fig. 1) were collected with the aid of butterfly nets or by hand from four different sites of YSR District (Lat. 14°28´N 78°49´E, 137 m Altitude), located in Andhra Pradesh state during February, 2013 to February, 2015. The collection sites include:

Site 1: Industrial Estate area (Lat. 14°47´N 78°76´E, 138 meters Altitude), YSR District,

Site 2: Campus area of Yogi Vemana University (Lat.14°28´N 78°49´E, 137 m Altitude), located in YSR District of Andhra Pradesh,

Site 3: Ramapuram village (14.05°N 78.75°E, 143 meters) Raychoti Mandal,

Site-4: Bouinpalli village, YSR District (Lat.14^{o}28'N 78^{o}52'E, 379 meters).

Host samples of different sizes i.e., small, medium and large were transported to laboratory and were instantly scrutinized after being euthanized.

2.1.1 Parasitological examination

The parasitological examination was carried out by separating various parts of the GI tract- oesophagus, stomach, intestine and rectum and the contents from each part was scraped into a petridish filled with 0.7% solution or Ringer's solution. These tissues were also teased out with needles to observe the helminths under the stereozoom microscope (LM-52-3621 Elegant). These helminth parasites were normally found attached to intestine of the host. Permanaent slides were prepared by the standard protocols i.e., Parasite fixation in A.F.A (Alcohol-85%, Formalin-5%, Glacial acetic acid- 10%) for 24 hours followed by staining with alum caramine, washing with water, dehydration using a graded sequence of alcohols (70%, 80%, 95%, 100%), clearing in xylol and finally mounting in Canada balsam or DPX mount onto a glass slide with coverslip Hiware et al., [35]. The

stained parasites were observed and identified under the Lynx trinocular microscope (N-800M) and their microphotographs were captured and line diagrams were drawn with the aid of attached drawing tube.

2.1.2 SEM studies

Parasites were fixed in 2.5% glutarldehyde in 0.1 M phosphate buffer (pH 7.2) at 4°C for 1 hour for SEM examination. They were washed in the same buffer before post-fixation in 1% osmium tetroxide in the same buffer at 4°C for 1 hour followed by dehydration through graded series of alcohols (70%-100% at 5-10 min interval), critical point dried and sputter coated with gold. SEM photographs at various magnifications were captured with a Carl Zeiss Scanning electron microscope (ΣIGMA TM) facility at Sri Venkateswara University, Tirupati. Identifications were made according to Yamaguti [36].

2.1.3 Pathological examination

The three parts of the gastro intestinal tract- stomach, intestine and rectum were fixed in 90% formalin and uninfected (control) organs were fixed separately to study the comparison between control tissue and infected tissues. However**,** of the three examined tissues i.e., stomach, intestine and rectum, only intestine of *H. tigerinus* was found to be infected with the digenean, *Tremiorchis ranarum* (Fig. 2). Intestine is the major organ for the parasitization as it plays a key role in digestion, absorption of food and production of gastro intestinal hormones. The fixed intestine samples were washed with water followed by graded series of alcohol dehydration (70%, 90%, 95% and 100%), clearing in xylol, infiltration with paraffin wax at 58°C and finally blocks were prepared for the section cuttings. Longitudinal and transverse sections of 4-8 µ thickness were prepared with the aid of Yorco YSI 115 rotary microtome to get the exact location of the parasite, the damage caused at a particular site of the tissue and the change in the chemical nature of the tissue of the organ affected. Sections were dewaxed and hydrated through descending grades of ethanol to water. They were initially stained in haematoxylin. Slides were dehydrated in 95% alcohol and stained in 10% alcohol eosin, cleared in xylol and mounted in Canada balsam.

3. RESULTS

The small intestine of the adult specimens of *H. tigerinus* were found to be parasitized by *Tremiorchis ranarum* (Digenea: Plagiorchiidae) reaching a prevalence rate of 11.3% (34/300). A total of 960 *T. ranarum* (range=1-299) with a mean intensity of 28.2 and mean abundance of 3.2 were recovered from these infected hosts. The incidence of *T. ranarum* infection at Industrial Estate, YSR District is was high (66.12 %) and least in Bouinpalli Village (4.6%) among the four study sites of YSR District. There is a weak correlation between host snout length and parasitic abundance ($r=0.0652$, $R^2= 0.0043$; $r_s= 0.1063$, $t= 1.85$). The number of parasites in males and females *H. tigerinus* were compared (Mann-Whitney U-test, Z-score= 0.24, $p= 0.405$ at $p<0.05$) and no significant differences were observed.

3.1 Taxonomic Summary

Parasite: *Tremiorchis ranarum* Mehra et Negi, 1926 belonging to family Plagiorchiidae Luhe, emert Ward, 1917

Type host: Indian bull frog *Hoplobatrachus tigerinus* belonging to Dicroglossidae family

Site of infection: Intestine

Host Locality: YSR District (Lat. 14°28´N 78°49´E, 137 m Altitude), located in Andhra Pradesh state.

Prevalence of infection: 11.3% (34 out of 300 examined frogs)

Material deposition: Voucher specimens deposited in the Department of Zoology, Faculty of Life science, Yogi Vemana University, YSR District, Andhra Pradesh, India.

3.2 Microscopic Examination (Figs. 3 & 4):

Body elongated 2.2-4.10 × 0.47-1.07, with small backwardly directed spines extending from anterior end to little posterior to hind end of the posterior testis. Cuticle thick and smooth. Oral sucker 0.12-0.35 × 0.14-0.35. Prepharynx small, thin walled; pharynx 0.1-0.15 × 0.1-0.15. Fore body 1.1-2.5 × 0.7-1.20; hind body 1.1-2.5 × 0.55-0.95. Intestinal caeca simple extending up to anterior or hind end of anterior or hind end of anterior testis. Acetabulum 0.15-0.25 × 0.14-0.2. Testis two, anterior testis 0.3-0.37 × 0.2-0.42, posterior testis, 0.3-0.32 × 0.2-0.45. Cirrus sac 0.54-0.55 × 0.14-0.15.Ovary 0.20-0.28 × 0.16-0.26. Vitelline follicles 0.05-0.11 × 0.25-0.10.Uterus small on posterior region. Eggs small and non-operculated 0.0125-0.075 × 0.010-0.015.

3.3 SEM Studies (Fig. 5)

Body oval in shape, tapering anterior side broadly rounded posteriorly, backwardly directed spines are present anterior to mid region. Oral sucker is round, sub-terminal. Ventral sucker is spherical.

3.4 Histopathogical Studies (Figs. 6-9):

In the present investigation, the histopathological changes in the tissues of the host *Hoplobatrachus tigerinus* in response to the infection of *Tremiorchis ranarum* was undertaken (Figs. 3-5). Alimentary canal of the host consists of oral cavity, oesophagus, stomach, intestine, rectum and anus. But intestine was the only site of infection for *T. ranarum* in the present study. Intestine consists of four layers - *serosa*, *muscularis layer, submucosa* and *mucosa*. The outermost serosa layer of the digestive tract is thicker at the region of intestine. Serosa consists of connective tissue fibres, cells, blood vessels and nerves. Next to serosa is the muscularis mucosa consisting of inner circular and outer longitudinal muscles (Fig. 6). A thin layer of loose connective tissue consisting blood vessels and nerves acts as a basement membrane between the muscle layers. Third layer of intestine is submucosa, often referred to as 'sub-epithelial layer' is highly vascularized and extends into villi and lamnia propria. Mucosa is highly folded large circular folds called *plicae circularis*, most numerous present in the upper part of the small intestine. Next to plicae *circularis* are small folds and finger like projections called *villi* and apical surfaces called *microvilli* which are lining columnar epithelial cells. Between the villi, crypts are present called *crypts of Liberkuhn*, extending down to *Muscularis mucosae* (Fig. 7). *T. ranarum* was one of the dominant parasites infecting the wild anurans. Parasites not only bring changes in the morphology of the infected organ but also interfere with nutrition and metabolism of the host thus, adversely distressing the host. The effect of parasites on the host causes series of interactions which ultimately reduces the absorption and other metabolic process. The helminth parasites cause damage to the villi and other layers of the intestine with their adhesive organs (suckers) and its sucking action result in mechanical damage such as desquamation of the epithelium, focal necrosis and increase in number of fibroblasts at the attachment point. Inflammation of villi at the site of attachment in the intestine cause fibrosis related with hyperplasia and metaplasia. Increase in number of lymphocytes in the stratum granulosum and connective tissue layer was an indicative of inflammation (Fig. 8). Vacuolation of sub-mucous cells and proliferative changes lead degeneration of the layers of the intestine was observed (Fig. 8). Parasites cause the dilation of blood vessels of the sub-mucosa to some extent which results in degeneration of intestinal folds, shrinkage of villi and necrosis of epithelial cells (Fig. 9). However, muscularis layer does not show much damage. The pathological effects include an increase in the thickness and the damage to the mucosa. The decrease in the thickness of the muscular layer is a common feature that occurs in helminth infections. Helminth parasites not only transform the morphology of the infected organ but also injure the various systems of the host such as nervous system, circulatory system, glands of internal secretion, interfere with nutrition and metabolism, disturb the movements and secretory functions of the alimentary canal. These effects unfavorably lead to disease or death of the host.

4. DISCUSSION

The Indian bull frog *Hoplobatrachus tigerinus* is the largest frog native to Asia and reaches upto 15cm in length. To date, helminth parasites occurring in Indian bull frog were poorly investigated from Southern India. The present study was planned to enhance our knowledge about the helminthic fauna infecting bull frog by investigating one of the digenean parasites infecting frog specimens collected from YSR District, Andhra Pradesh. To accomplish this study, a total of 300 frog specimens were collected from the studied area and scrutinized for the parasitic infections. The present parasitological examination revealed that rate of the prevalence of parasitization of digenean parasites in the examined frog specimen was only 11.3% (34 out of 300 examined) which shows that parasitization was not so heavy in the infected frogs. The genus *Tremiorchis* was proposed by Mehra et Negi [37] with *Tremiorchis ranarum* as its type species from the small intestine of *Rana tigrina.* Bhalerao [38] described the same form as *Centrovitus pentadelphi* which was later synonimized as *T. ranarum by* Verma [39]. *Tremiorchis* species were reported from frogs, toads and reptiles by many scientists Verma, [39]; Singh, [40]; Bharadwaj, [41]; Agarwal, [42]; Ali and Karyakarte, [43]; Dwivedi and Chauhan, [44]; Mukerjee and Gosh, [45]; Sinha and Sahay, [46], Pandey, [47]. Later, there were few more species added to the genus i.e, *T. varanum*; *T. mehrai*; *T. vitelloconfluentum*; *T. tigrinum*; *T. mathuraensis* and *T. spiniphlyctis*. But all these species were considered to be the synonyms of *T. ranarum*. Pandey and Agrawal [48] added a note on the status of the genus *Tremiorchis*. Pandey et al., [49] studied the morphological intra specific variations of *T. ranarum.* Extensive literature revealed that there are many studies on the nature of helminth infections, but information on the pathogenic effects, particularly in wild caught frogs remains completely meager. A meager amount of work was done on histopathology of intestine in *Hoplobatrachus tigerinus* Boon and Kok, [50]; Login and Dvorak, [51]; Pressnell, [52]; Ouellet, [53]. In the present survey, the histopathological changes in the intestinal tissues of the host *Hoplobatrachus tigerinus* revealed that the tissue damage is prominent with wide spread epithelial necrosis, inflammation of villi with hyperplasia and metaplasia, dilation of blood

vessels, increased number of lymphocytes in the stratum granulosum and connective tissue layer. Similar trend was noticed by Khatoon [54], Benarjee and Reddy [55], Bamidele [56], Hossain et al., [57]. Similarly, Raissy and Ansari [19], Kurpad [58], Pardeshi et al., [59]; Rezaei et al. [60], Shareef [61] and Vankara [62] observed the histopathological changes in the various tissues of different vertebrates.

Fig.1

Fig.2

Fig.3

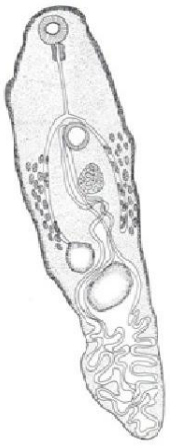

Fig.4

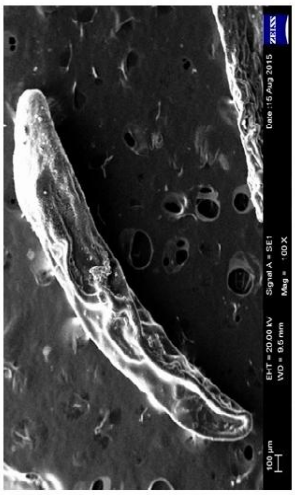

Fig.5

Fig.1: *Hoplobatrachus tigerinus*; Fig.2: *Tremiorchis ranarum* recovered from the intestine of *H. tigerinus*; Fig.3: Microphotograph of *T. ranarum*- 100X; Fig.4: Line diagram of *T. ranarum*- 40X; Fig.5: SEM photograph of *T.ranarum*-100µm

Fig.6

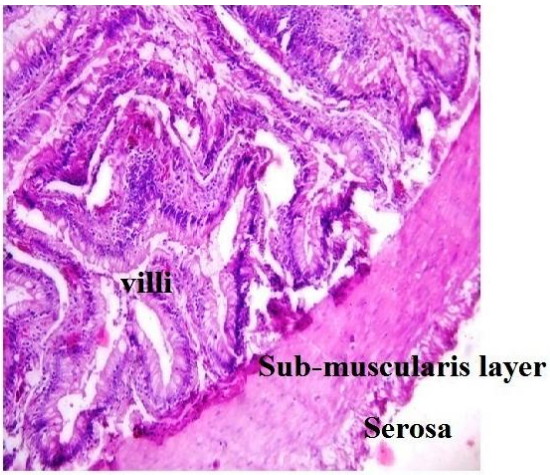

Fig.7

Fig.8

Fig.9

Fig.6: T.S. of uninfected intestine- 40X; Fig.7: T.S. of uninfected intestine- 100X; Fig.8: T.S. of infected intestine with *Tremiorchis ranarum*- 40 X; Fig. 9: T.S. of infected intestine with *T. ranarum*- 100X

5. CONCLUSION

During the taxonomic evaluation on the metazoan parasites of amphibians of YSR District, *H. tigerinus* was found to be severely infected with the parasite *Tremiorchis ranarum* which immensely captivated to study the pathological changes induced by these parasites on the host. Hence, the pathological changes in the intestine induced by *Tremiorchis ranarum* at the site of attachment were studied carefully. The histopathological changes in the intestinal tissues of the host *H. tigerinus* revealed that the tissue damage is prominent with wide spread epithelial necrosis, inflammation of villi with hyperplasia and metaplasia, dilation of blood vessels, increased number of lymphocytes in the stratum granulosum and connective tissue layer.

SIGNIFICANCE STATEMENT

This study discovers the fact that the parasite, *Tremiorchis ranarum* causes a lot of mechanical damage to the intestine of the host. These parasites in huge numbers can affect the fish health and its

productivity. These types of studies will definitely help the future researchers in this area to gain knowledge about the pathological changes induced by the parasites on the host tissues.

ETHICAL APPROVAL

All procedures contributing to this work comply with the ethical standards of the relevant national guides on the care and use of laboratory animals and have been approved and authorized by IAEC (Institution of Animal Ethics Committee-Regd. No.1460/PO/a/11/CPCSEA, dt. 20.05.2011), Zoology Department in Faculty of Life Sciences, Yogi Vemana University, Andhra Pradesh.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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