Shiraz University

IJVR

ISSN: 1728-1997 (Print)
ISSN: 2252-0589 (Online)

Vol. 22 No.1 Ser. No. 74 2021

IRANIAN JOURNAL OF VETERINARY RESEARCH

Original Article

Molecular characterization and histo-physiological alterations induced by concurrent helminthosis in the liver of urban commensal rodents in Punjab, India

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10.22099/ijvr.2020.38050.5539

(Received 8 Aug 2020; revised version 13 Oct 2020; accepted 9 Nov 2020)

Abstract

Background: Rodents harbour a number of parasites of public health importance, thus, they threaten human health and livestock. **Aims:** The present study aimed to characterize two helminthic species found in commensal rodents and record histo-physiological alterations induced by them. **Methods**: A total of 300 synanthropic rodents of three species: *Rattus rattus* (n=201), *Bandicota bengalensis* (n=90), and *Mus musculus* (n=09) were live trapped and necropsied in different seasons during November 2017 to October 2019 at Ludhiana, Punjab, India. **Results:** Liver of two species *B. bengalensis* (72.22%) and *R. rattus* (65.67%) were found infected with two helminthic parasites *Taenia taeniaeformis*, and *Calodium hepaticum*. These endoparasites were present either alone (4.33-6.33%) or as mixed infection (65.55%). The level of total proteins and liver marker enzymes including aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were found significantly higher in the liver of rodent species infected with single and mixed infection compared to those with no infection. In histopathological assay, granulomatous liver lesions and necrosis of hepatocytes were seen which were associated with eggs and adults of *C. hepaticum* and inflammatory reaction in hepatic parenchyma adjoining to cysts of *T. taeniaeformis*. Based upon scanning electron microscopy (SEM) identification and molecular characterization using mitochondrial cytochrome oxidase I (COI) region, the metacestodes in whitish cysts were confirmed to be of *T. taeniaeformis* for the first time in Punjab, India. **Conclusion:** The study highlights an alarmingly high infection of rodents with zoonotic parasites and suggests immediate pest (rodent) control to check the dissemination of zoonotic diseases by helminth species under study.

Key words: Helminthosis, Molecular characterization, Morphology, Pathophysiology, Zoonotic diseases

Introduction

Rodents act as a vital component in various ecosystems either by acting as a prey to its predators or as a carrier and reservoir of the zoonotic diseases (Okoye and Obiezue, 2008; Singla *et al*., 2008b; Singla and Singla, 2019). They also harbour a number of ecto- and endo-parasites of public health importance thus posing a threat to human health while living in close proximity (Singla *et al*., 2008a; Kataranovski *et al*., 2010; Mohd Zain *et al*., 2012). Hence increased rodent population in an area could be directly related to increased zoonotic diseases in human population (Stojcevic *et al*., 2004).

The parasitic fauna of rodents has been studied (Fagir and El-Rayah, 2009) in order to achieve control or eradication of parasites and also considering the important role of these animals in scientific research. Besides, some of these parasitic infections can induce physiological and immunological alterations in the hosts by causing severe tissue damages, stimulating abnormal growth, competing with host nutrients, decreasing the volume of host's blood and body fluids and by mechanical interference (Aboel-Hadid and Allam, 2007).

Adult worms of *Calodium hepaticum*, a nematode parasite of wild rodents and other mammals inhabit the liver. The female worms deposit clusters of eggs in the liver parenchyma, which become encapsulated as a result of the chronic inflammatory response of the host (Mowat *et al*., 2009; Singla *et al*., 2013). The distribution of *C. hepaticum* is ubiquitous and has been recorded throughout Europe, North and South America, Africa, Asia and Australia (Spratt and Singleton, 2001).

Taenia taeniaeformis is the commonly occurring intestinal tapeworm of cats and related carnivores whose larval stage, *Cysticercus fasciolaris* is found in liver of rats and mice (Moudgil *et al*., 2013). Rodents serve as intermediate hosts and are infected by ingesting the ova in contaminated food and bedding materials (Jithendran and Somvanshi, 1999). *Taenia taeniaeformis* infection is clinically asymptomatic and is considered harmless

(Singla *et al*., 2003).

The helminth fauna of the rodents in different countries has been well documented, whereas our knowledge on the helminth communities in rodents of India is patchy. Studies on helminth parasites of rodents are important because of their potential as reservoir of zoonotic infections and from the view point that they are vectors of serious diseases to livestock and laboratory animals (Easterbrook *et al*., 2008). Though few studies on the prevalence of *C. hepaticum* and *T. taeniaeformis* have been conducted from India, morphological and molecular characterization is lacking. The present study was done on molecular confirmation and morphological characterization of *T. taeniaeformis* along with its pathophysiology alone and in combination with *C. hepaticum*.

Materials and Methods

Collection and examination of rats

A total of 300 individuals of three commensal rodent species, namely the house rat, *Rattus rattus*, the lesser bandicoot rat, *Bandicota bengalensis*, and the house mouse, *Mus musculus*, were live-captured from three different habitats *i.e.* residences/street food shops, poultry farms and fish market using single- and multi-catch traps between November 2017 and October 2019 at Ludhiana, Punjab province of India. Traps were placed along the walls and on rodent runways. Bread (Indian chapatti) pieces were used in the single-catch traps and wheat grains in multi-catch traps as a lure for rodents. After trapping, all the animals were brought to the laboratory on the same day. They were kept individually in laboratory cages for 5-10 days and were provided with food and water *ad libitum* prior to autopsy.

Approval was obtained from the Institutional Animal Ethics Committee of Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana, India for use of animals vide memo No. IAEC/2018/1153-1188 under Protocol No. GADVASU/2018/IAEC/46/16. The procedures used in this study adhered to the guidelines of Committee for Control and Supervision of Experiments on Animals, India.

In the laboratory, all animals were necropsied to expose the liver via a midventral incision and inspected grossly for the presence of endoparasites and lesions (Singla *et al*., 2008b). Microscopic examination of cysts in liver tissue was performed by tissue press technique for the presence of parasites. Livers with cysts were collected in normal physiological saline, and then the numbers and dimensions of the cysts were recorded. Also, the liver tissues having the cysts were kept in 70% ethanol and 2.5% glutaraldehyde for molecular characterization and scanning electron microscopy (SEM) studies, respectively. The rats were categorized into three groups:

1) With single parasitic species infection
2) Mixed parasitic species infection
3) The animals without any infection

Scanning electron microscopy

Specimen preparation for SEM of *C. fasciolaris* was done at Electron Microscopy and Nanoscience Laboratory, Punjab Agricultural University, Ludhiana as per specific standard protocol. Parasites were kept overnight in 2.5% glutaraldehyde, washed thrice with 0.2 M rinsing buffer, kept in 1% osmium tetraoxide for 2-4 h, again washed thrice with 0.2 M rinsing buffer, dehydrated in ascending concentration of alcohol, gold coated, and mounted on the stub to obtain high quality scanning electron microphotographs to show different morphological features at electron accelerating voltage of 15 kV.

Biochemical estimation

Liver tissue (0.5 g) homogenized in 2 ml of phosphate buffered saline (PBS: 0.1 M, pH = 7.4) and centrifuged at 402 × g for 10 min was used to determine enzymatic activity. Total soluble proteins (TSP) (mg/g tissue) were estimated as per the method given by Lowry *et al*. (1951). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities (IU/L) (the most sensitive biomarkers directly implicated in the extent of hepatic damage and toxicity) were assayed as per the standard methods (Bergmeyer, 1974).

Histopathological study

A small portion of the liver (having yellowish white lesions and cysts) of infected rats was excised, fixed in Bouin's fluid, and processed into paraffin wax. Sections were cut, stained with haematoxylin and eosin (H&E) and studied under a light microscope.

Molecular characterization

Genomic DNA of the adult parasites (preserved in 70% ethanol) was extracted using QIAamp tissue kit (Qiagen, Hilden, Germany) as per the manufacturer's protocol with slight modifications. The parasites were mechanically disrupted by using sterile pestle-mortar. Final elutions of DNA were made in 20-100 µL of elution buffer.

For detection of *T. taeniaeformis*, the genomic ribosomal DNA extracted from the metacestodes was used in a polymerase chain reaction (PCR) to amplify the mitochondrial cytochrome oxidase subunit 1 (COI). The forward JB3 (5´-TTT TTT GGG CAT CCT GAG GTT TAT-3´) and reverse JB4 (5´-TAA AGA AAG AAC ATA ATG AAA ATG-3´) primers were used which are considered to be the universal primers (Bowles *et al*., 1995). The PCR protocol was as follows: initial denaturation for 5 min at 95°C followed by 35 cycles of denaturation for 45 s at 95°C, annealing for 45 s at 52°C, and extension for 45 s at 72°C, and after 35 cycles a final extension step for 5 min at 72°C. During each amplification reaction, a no template control was also included in each plate as negative control.

Amplification product was analyzed on 1.5% agarose gel and visualized by ethidium bromide staining. Polymerase chain reaction product was purified using QIAquick® PCR purification kit as per the

manufacturer's protocol. The purified PCR product was sequenced at Xcleris Genomics, Ahmadabad, Gujarat, India. The nucleotide sequence obtained from the PCR product was put into basic local alignment search tool (BLAST) and compared with other available cyclophyllidean cestode sequences (of accession No. EU544579, AB465239, AM503325, FJ939135, EU544598, EU544558, AM503316, AM503330, EU544595, AB221484, EU544597, EF090612, EU544596, KF702312, KT693090, and EU544580) from GenBank using BLAST (2.2.22). Then, the sequences were aligned using ClustalW pairwise alignment with the default gap. The phylogenetic tree was constructed using the Maximum Parsimony method in MEGA X (Felsenstein, 1985; Kumar *et al*., 2018). Branch support was given using 1000 bootstrap replicates.

Statistical analysis

The significance of difference in biochemical parameters among rodents having no infection and those with single and mixed infections was determined based on one-way analysis of variance (ANOVA) using SPSS (statistical package for social sciences) software. All pair-wise treatment comparisons were made using Tukey's (honestly significant difference) test at 5% level of significance.

Results

Out of a total of 300 rodents examined, livers of 197 (65.67%) were found infected with two different helminthic parasites i.e., *T. taeniaeformis* and *C. hepaticum* alone (6.33 and 4.33%, respectively) and in concurrence (65.55%) with each other (Table 1). Out of 201 *R. rattus*, 90 *B. bengalensis*, and nine *M. musculus* individuals examined, the rate of infection was highest in *B. bengalensis* (72.22%) followed by *R. rattus* (65.67%) (Table 1). No infection was found in *M. musculus*.

Morphological characters

The metacestode of *T. taeniaeformis* was observed in whitish raised single to multiple (1-6) parasitic cysts in the liver (Fig. 1A) of two rodent species, with a higher overall prevalence in *B. bengalensis* (68.89%) followed by *R. rattus* (60.69%) (Table 1). The size of the cysts varied from 4 to 12 mm in diameter. Each cyst contained a single, live, characteristic larva measuring 10 to 18 cm in length.

Gross lesions comprised of irregular yellowish white lesions or streaks were found randomly scattered on the liver surface of 117 *R. rattus* and 61 *B. bengalensis* rats (Fig. 1B). On further investigation, it became clear that these rats were infected with eggs/worms of *C. hepaticum*. The higher prevalence of *C. hepaticum* was recorded in *B. bengalensis* (67.78%) than in *R. rattus* (58.20%) with an overall prevalence of 59.33% in both the species (Table 1). Out of these infected rats, 107 (53.23%) *R. rattus* and 58 (19.33%) *B. bengalensis* had mixed infection of *T. taeniaeformis* and *C. hepaticum* (Table 1, Fig. 1C).

The SEM view of metacestodes of *T. taeniaeformis* revealed scolex with hooks and lateral suckers, a portion of the strobila showing segmentation, and the terminal bladder like end (Figs. 2A-C).

Table 1: Prevalence of different parasitic infections in different rodent species

Host species	Sample size (%)	Infected animals (%)	Helminth parasites			
			T. taeniaeformis		*C. hepaticum*	
			Single infection (%)	Mixed infection* (%)	Single infection (%)	Mixed infection* (%)
Rattus rattus	201 (67.00)	132 (65.67)	15 (7.46)	10 (4.97)	107 (53.23)	10 (4.97)
Bandicota bengalensis	90 (30.00)	65 (72.22)	04 (4.44)	03 (3.33)	58 (19.33)	03 (3.33)
Mus musculus	09 (3.00)	-	-	-	-	-
Total	300	197 (65.67)	19 (6.33)	13 (4.33)	165 (55.00)	13 (4.33)

* Mixed infection with *C. hepaticum* and *T. taeniaeformis*

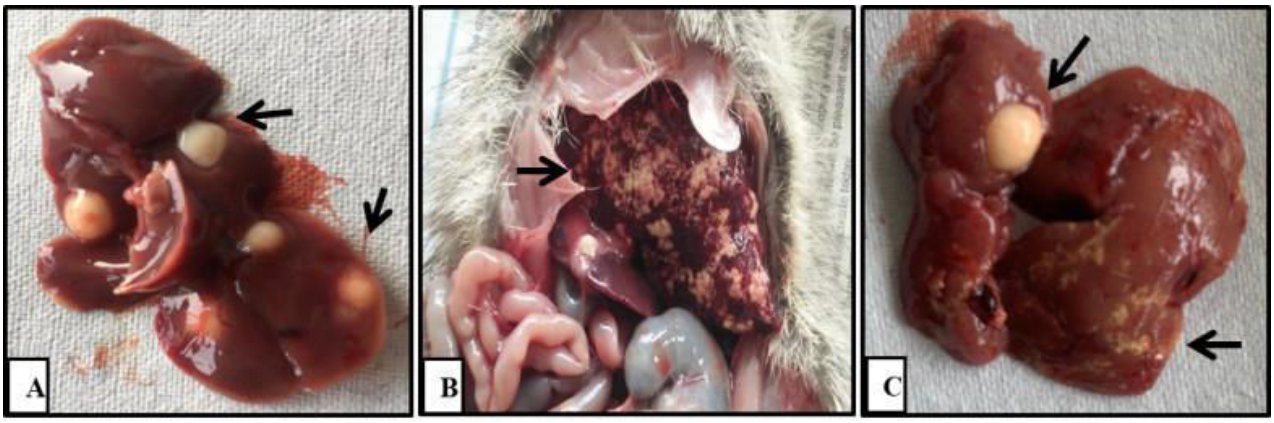

Fig. 1: Gross photographs of liver showing severe infection of helminth parasites. *Taenia taeniaeformis* infection in the form of a number of whitish cysts (arrows) (**A**), *C. hepaticum* infection in the form of pale lesions (arrow) (**B**), and mixed infection of *C. hepaticum* in the form of pale lesions (arrow) and *T. taeniaeformis* in the form of whitish cyst (arrow) in the same liver (**C**)

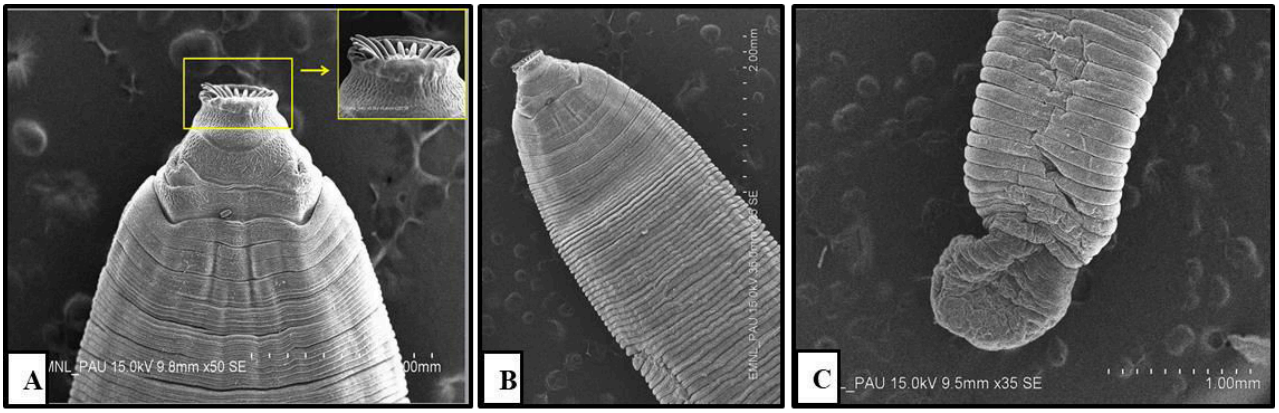

Fig. 2: Scanning electron microphotographs of *T. taeniaeformis* larva. Scolex with a row of hooks and lateral suckers (**A**), strobila with segmentation (**B**), and the terminal bladder like posterior end (**C**)

Table 2: Effect of parasitic infections on the level of TSP and activities of liver marker enzymes (ALT and AST) in liver of *R. rattus* and *B. bengalensis*

Parameters	*R. rattus*			*B. bengalensis*		
	No infection	Single infection	Mixed infection	No infection	Single infection	Mixed infection
TSP	7.22 ± 0.56^{b}	11.82 ± 1.28^{a}	20.02 ± 0.40^{a}	8.17 ± 0.16^{b}	12.69 ± 1.47^{b}	21.27 ± 0.34^{a}
AST	36.02 ± 3.77^{b}	52.51 ± 1.00^{a}	60.97 ± 3.50^{a}	35.97 ± 3.40^{b}	55.88 ± 1.72^{a}	62.36 ± 1.65^{a}
ALT	19.87 ± 2.06^{b}	30.77 ± 2.26^{a}	30.04 ± 2.02^{a}	22.06 ± 1.30^{b}	31.27 ± 0.9^{a}	33.33 ± 0.19^{a}
AST/ALT	1.80	1.70	2.02	1.60	1.80	1.90

Values are mean±SE, TSP: Total soluble proteins (mg/g), AST: Aspartate aminotransferase (IU/L), and ALT: Alanine aminotransferase (IU/L). Mean values with different superscripts ($^{a, b}$) in a row for different rodent species and biochemical parameters differ significantly (P<0.05)

Biochemical analysis

Compared to the control group, the level of TSP increased significantly (P<0.05) in the liver of infected *R. rattus* and *B. bengalensis* (Table 2). In addition, the levels of ALT and AST activities were increased significantly (P<0.05) in the liver of both the infected groups of *R. rattus* and *B. bengalensis* (Table 2). The De Ritis ratio (AST/ALT ratio) in *R. rattus* species was elevated in rats infected with mixed infections i.e. 2.02 as compared to rats with single infection (1.70) and no infection (1.80) (Table 2). Whereas, in *B. bengalensis*, it was elevated in both the infected groups i.e., single infection (1.80) and mixed infection group (1.90) as compared to uninfected rats (1.60) (Table 2).

Histopathological alterations

The larva of *T. taeniaeformis* was found encapsulated by connective tissue capsule (Figs. 3A and B). The cyst wall was thick, surrounded by inflammatory cells, mostly lymphocytes. The liver parenchyma immediate to cyst showed necrotic changes. Fatty change along with granular degeneration and atrophy was evident in the adjacent liver parenchyma and hepatocytes surrounding the cysts. There was marked infiltration of lymphocytes along with congestion and dilation of sinusoids and congestion of central hepatic veins (Figs. 3C and D). Despite the marked hepatic tissue damage owing to severe capillariasis and larval infection of *T. taeniaeformis* found in present the study, all the rats appeared healthy and active.

In the present study, large deposition of eggs was found in the liver tissue of rats infected with *C. hepaticum*. Adult and gravid female worms with eggs in the uterus (Figs. 4A and B) along with dead and disintegrating parts of adult worms were also observed in the liver tissue sections. The adult worms and eggs of *C. hepaticum* were surrounded by mixed population of inflammatory cells including eosinophils, macrophages, occasional lymphocytes and plasma cells with necrotic debris. Typical eggs with bipolar caps were found scattered in the parenchyma of the liver, predominantly in portal areas and granulomatous reaction was seen around them giving rise to several microgranulomas or coalesced macrogranulomas (Figs. 4C and D). The microgranulomas consisted of aggregates of eggs surrounded by mature fibrous connective tissue containing a few lymphomononuclear cells. Additionally, the surrounding parenchyma showed granular degeneration and adjacent parenchyma revealed congestion, necrosis, and microgranulomas (Fig. 4D).

Molecular characterization of metacestode

The mtCOI region of the metacestodes was successfully amplified. Polymerase chain reaction amplification of the region showed a single band of approximate size of 473 bp (Fig. 5). The BLAST showed that the sequences of the metacestode are closer to those of species of *Taenia*, with maximum similarity (of about 93%) to *T. taeniaeformis* (accession No. EU544596). Multiple alignment of COI of query sequences with three different isolates shows the presence of 1.3% mismatches with no gap (Fig. 6). According to the

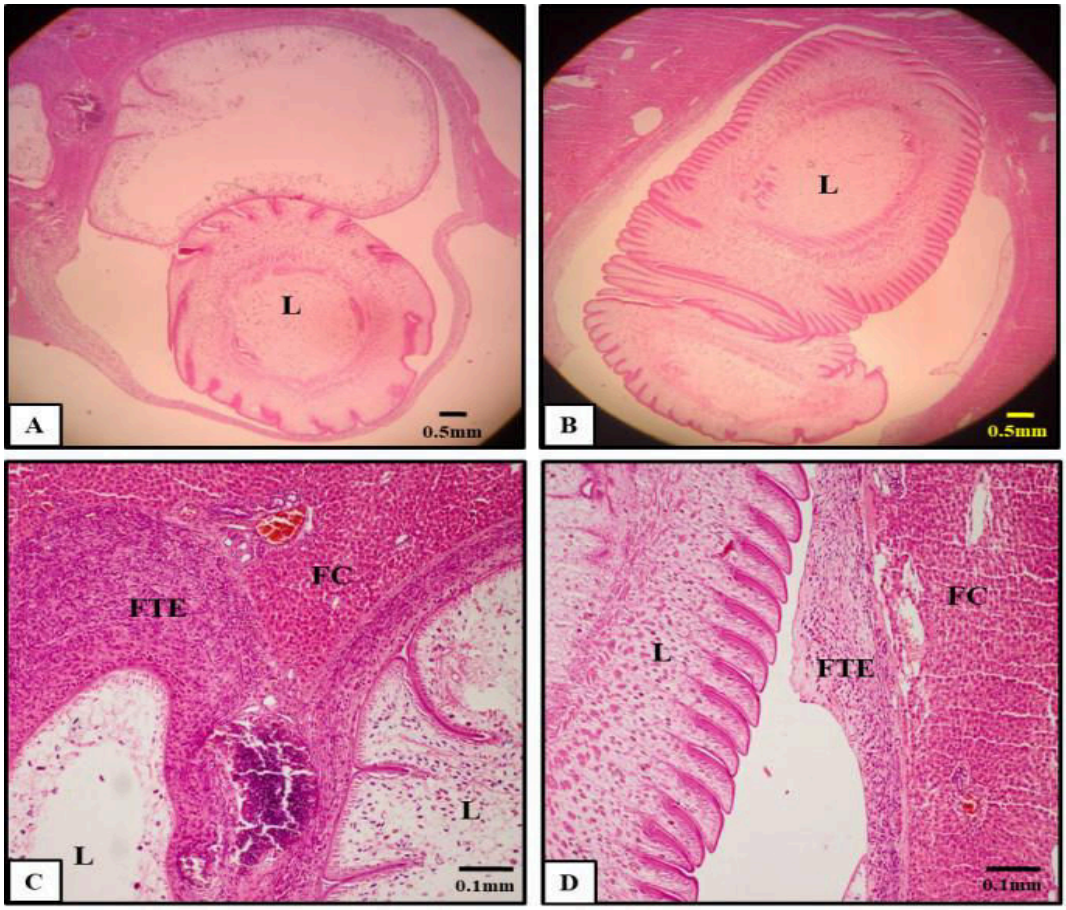

Fig. 3: Haematoxylin and eosin stained sections of liver showing *T. taeniaeformis* infection. Presence of *T. taeniaeformis* larva (L) encapsulated in fibrous tissue (FTE), and severe degeneration and fatty change (FC) in the adjacent liver parenchyma; Original objective, ×4 (**A**, **B**) and ×10 (**C**, **D**)

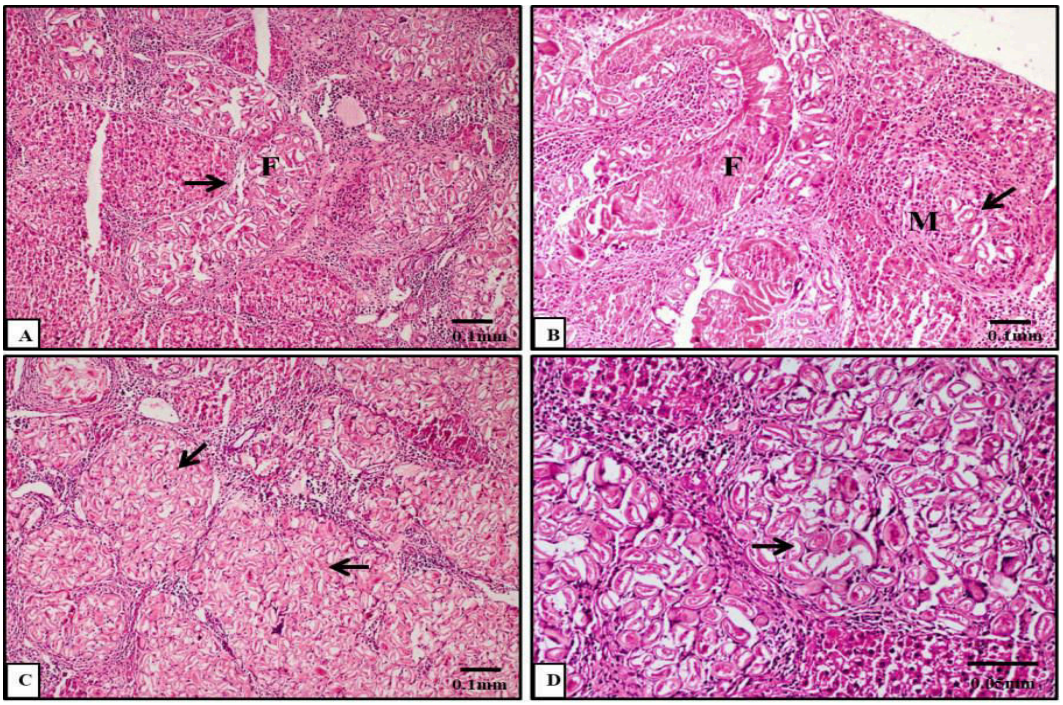

Fig. 4: Haematoxylin and eosin stained sections of liver showing severe infection of *C. hepaticum*. Adult and gravid female *C. hepaticum* (F) surrounded by macrogranuloma (M) containing eggs and macrophages in the portal area (arrow); Original objective ×10 (**A**, **B**), and necrosis of hepatocytes with the presence of encapsulated bipolar eggs (arrow); Original objective ×10 (**C**) and ×40 (**D**)

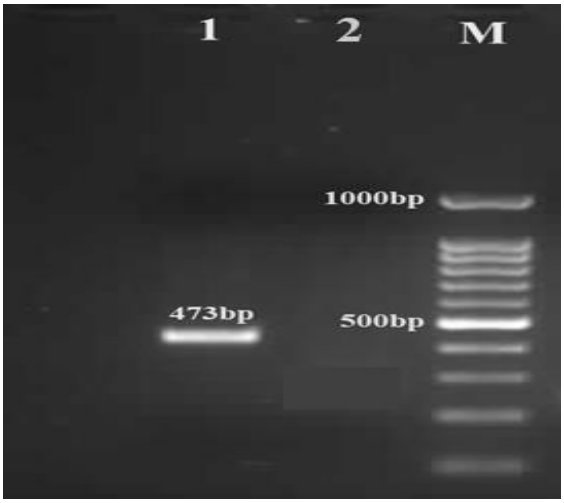

Fig. 5: The PCR amplification of COI region of metacestode. Lane M: 100 bp DNA ladder. Lane 1: Positive sample with band size of 473 bp, and Lane 2: Negative control

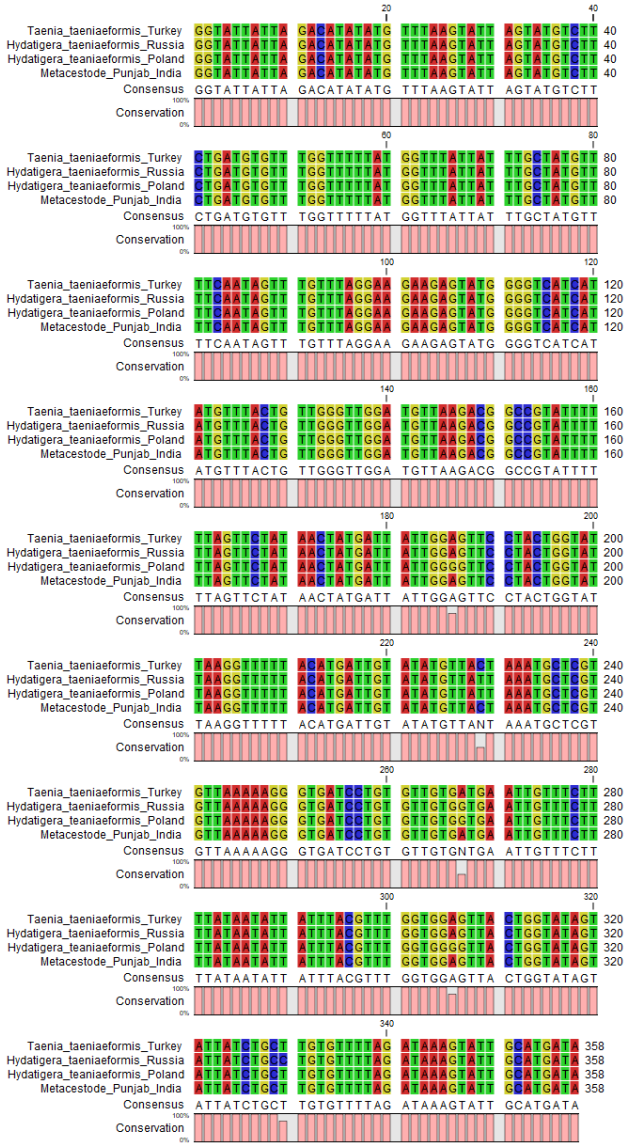

Fig. 6: Multiple alignment of the query metacestode sequence with other close taeniid cestode sequences

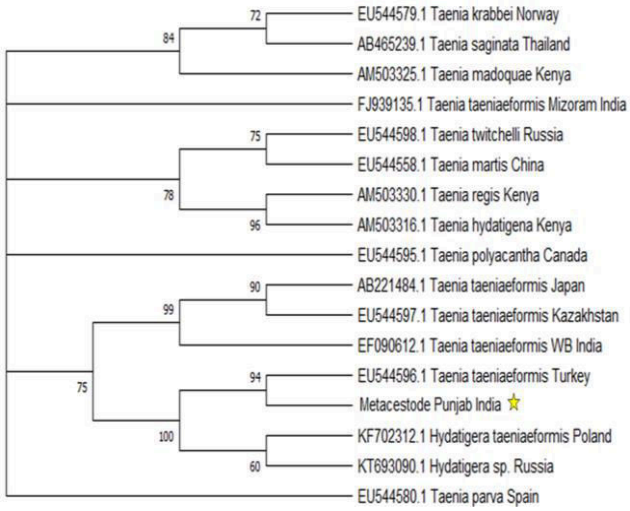

Fig. 7: Phylogenetic tree showing the relationships between the present metacestode samples and previously reported *Taenia* species based on the COI region constructed using Maximum Parsimony method. The numbers on the branches refer to the bootstrap values

phylogenetic tree, the present sequences are placed in the same clade with *T. taeniaeformis* isolates from Turkey and these two are placed in the same clade with *Hydatigera taeniaeformis* isolates from Poland and Russia (Fig. 7). The Nucleotide sequence data reported here have been submitted to the GenBank with the accession number LC585870.

Discussion

Hepatic capillariasis, a disease caused by *Capillaria hepatica* (now called *C. hepaticum*) is common in rodents but is rare in humans. Seventy-two cases in humans have been reported worldwide since the first case was described by MacArthur in 1924 (MacArthur, 1924; Fuehrer *et al*., 2011). Out of 490 residents in the urban area of Porto Velho, Brazil, 1.8% were found infected with *C. hepatica* (Rocha *et al*., 2015). Another example is a case report of a severe infection in a 5-year-old child of Iran (Aghdam *et al*., 2015). In wild rodents, the eggs of *C. hepatica* may be liberated from the liver into the external environment (soil/water) by the natural death of the host and decomposition of the body, from where it can infect humans and other animals (Spratt and Singleton, 2001).

Similar to the present study, Zamini *et al*. (2017) recorded 50% each of *R. rattus* and *R. norvegicus* infected with *C. hepatica* in Iran. The infection rates of *C. hepatica* in some other parts of the world were, 36% in *R. norvegicus* in Milan, Italy (Ceruti *et al*., 2001), 8% in three non-commensal rodent species from Geneva, Switzerland (Reperant and Deplazes, 2005), 19.1% in 2 invasive species of wild rats in Malaysia (Tijjani *et al*., 2020) and 87.9% in Norway rats in Baltimore, USA (Easterbrook *et al*., 2007) which are comparable to our study. Genus *Rattus* is considered as a reservoir host for this infection; infected rodents are hence reported as a

risk for public health (Zamini *et al*., 2017).

Another study conducted by Resendes *et al*. (2009) reported *Capillaria* infection in the liver of *M. musculus* and concluded that house mice also act as a source of infection, similar to *R. rattus*. However, the study showing the low prevalence of *C. hepatica* in *M. musculus*, has concluded that this species is less affected by *C. hepatica* (Zamini *et al*., 2017). Results of the present study also showed no infection of *C. hepaticum* in house mice, which could infer that house mice probably play no active role in the life cycle of this disease in Punjab State of India.

Based on the morphological similarities, supplemented by close matching of mtCOI sequence of the metacestode under study with that of *T. taeniaeformis*, it can be concluded that the parasite recovered from the liver cysts of rodents in the present study area indeed represents larva of *T. taeniaeformis*, the adult of which occurs in the various carnivorous animals that commonly infect the rodent host.

Taenia taeniaeformis has been found in the small intestine of cats and the cysticercus stage occurs in rats (Singla *et al*., 2009). Human infection with this parasite was first reported in a 5-year-old boy from Buenos Aires by Bacigalupo (1922). In humans, there are a number of records of the occurrence of adult (Ekanayake *et al*., 1999; Moudgil *et al*., 2013) and larval (Sterba *et al*., 1977) forms of *T. taeniaeformis*. However, this cestode is considered to pose a low health risk. Kobayashi *et al*. (2013) also reported disseminated cysticercosis concurrent with taeniosis in a 31-year-old male Japanese traveler after returning from India.

In the intermediate host, the hepatic function may be disrupted by excessive development of the cysticerci and degenerative fatty changes, and thus the animal may die. However, natural cysticercus infection is asymptomatic and rats are considered harmless (Jithendran and Somvanshi, 1999).

Jithendran and Somvanshi (1999) recorded natural incidence and pathology of *T. taeniaeformis* infection in laboratory rats and mice and found that the source of infection was contamination of *T. taeniaeformis* eggs in bedding material by cats. The occurrence of *C. fasciolaris* has been reported in a laboratory and wild rodent species by many workers in India (Singla *et al*., 2003; Singla *et al*., 2016) as well as in other countries (Paramasvaran *et al*., 2009; Mcinnes *et al*., 2014; Herawati and Sudarmaji, 2019; Tijjani *et al*., 2020) but no molecular characterization was conducted.

Similar to the present study, a high prevalence of metacestodes of *T. taeniaeformis* was also recorded in the lesser bandicoot rat in Bangladesh (Fuehrer *et al*., 2012). The strobila of the metacestode reported by Fuehrer *et al*. (2012) was armed with 28-30 hooks, whereas in the present study, the strobila was armed with 36-38 hooks.

The activities of ALT and AST enzymes are the most sensitive biomarkers directly implicated in the extent of hepatic damage and toxicity (El-Demerdash, 2004). Transaminases are involved in transferring the amino groups of aspartate and alanine to ketoglutaric acid. Hepatocyte injury results in altered cell membrane permeability causing excessive leakage of transaminases (Kasarala and Tillmann, 2016). The significantly elevated levels of AST and ALT in the liver of infected rodents suggested that *C. hepatica* and *C. fasciolaris* alone and in mixed infection caused severe toxicity and damage in the liver hepatocytes. Research conducted by Botros and Sikaris (2013) also suggests that, when body tissues are damaged, AST and ALT are released into the bloodstream and increase the serum enzyme level.

The AST/ALT ratio, also known as the De Ritis ratio, is useful in assessing various liver diseases (Bhelonde and Ghosh, 2002). An elevated level of AST/ALT ratio is predictive of long term complications including fibrosis and cirrhosis in the liver tissue (Botros and Sikaris, 2013), which is in accordance to the present study, where the liver of infected rats (single and mixed infection) showed increased AST/ALT ratio.

The microscopic observations of liver sections infected with the larva of *T. taeniaeformis* observed in this study provide a correlation with the biochemical findings. The tissue changes have been more clearly depicted than the earlier reports (Jithendran and Somvanshi, 1999; Singla *et al*., 2013; Singh and Arya, 2015). The worms of *C. hepaticum* were round, had an eosinophilic wall and their reproductive system was clearly recognizable describing the changes in a better manner in the present natural infection when compared with previous reports (Mowat *et al*., 2009; Singla *et al*., 2013; Zamini *et al*., 2017).

The present study thus reports pathological and biochemical changes associated with the single and mixed infection of helminth parasites of zoonotic importance which naturally infect urban commensal rodents thus pose a health risk to humans and other domestic animals.

Acknowledgements

Authors are thankful to the Indian Council of Agricultural Research, New Delhi, India for providing funds in the form of All India Network Project on Vertebrate Pest Management (Rodent Control) at Punjab Agricultural University, Ludhiana, India. Authors are also thankful to the Department of Science & Technology for providing laboratory infrastructure in the form of FIST project.

Conflict of interest

Authors state no conflict of interest.

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