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Migratory behaviour and pathological changes of *Toxocara canis* in organs and tissues of experimentally infected Balb/c mice

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Abstract
Toxocariasis is an important parasitic zoonosis caused by the infective larvae of *Toxocara canis*. In the present study, the migration and pathological effects of *T. canis* larvae were evaluated after experimental infection of Balb/c mice with two doses of embryonated eggs. The mice were divided into 3 groups; Group I: infected with 100 doses; Group II: infected with 1000 doses; Group III: control uninfected mice. The mice were sacrificed at 7, 14, 21 and 28 days post infection for larval recovery and histopathology studies. The results showed that the maximum numbers of larvae were recovered from liver and lungs on 7 dpi and from the brain and muscle on 28 dpi. No larvae were found in kidneys and eyes of all the infected mice throughout the study. Histopathological examination revealed congestion, haemorrhage and inflammatory infiltrate in the lungs, liver and kidneys in both the infected groups between 7 and 28 dpi. The inflammatory cells present in all the infected tissues studied were lymphocytes, eosinophils and macrophages. In the brain, no inflammatory reaction was observed around the migrating larvae. Granulomas were seen around trapped larvae in the lung and liver but in the brain, only sections of larvae were observed on 28 dpi. In this study murine model were used which mimics the biology of human infection, might be relevant to a better understanding of human toxocariasis for future diagnostic and therapeutic studies.

Keywords: *Toxocara canis*, Balb/c, larval migration, histopathology, granulomas

1. Introduction
Toxocara canis is one of the most important gastrointestinal helminths of dogs, with infections reported from all parts of the world. Due to its zoonotic potential, this round worm is of special interest not only for veterinarians, but also for medical practitioners [1]. Human infections can be acquired by ingestion of embryonated eggs of *T. canis*, from soil via the faeces of infected dogs. After ingestion of eggs, the larvae penetrate the intestinal wall to migrate through the liver and other viscera, including the central nervous system [2]. Depending on the organs affected, the predominant symptoms are classified as visceral larval migrans, ocular larval migrans, neurologic and covert toxocariasis [3]. Most often the migrating larvae become encapsulated in a granulomatous response or accumulate in the brain, where they elicit little or no histological reaction [4].
Transmission of *T. canis* to dogs depends on several mechanisms; one of them includes the transfer of third stage larvae from paratenic host tissues to dogs by prey-predator relationship. A wide range of animals including mice, rats, rabbits, gerbils, monkeys and humans act as paratenic hosts [5]. Bush *et al*. [6] defined a paratenic host as a host in which development does not occur, but which may serve to bridge an ecological, or trophic, gap in a parasite's life cycle. Studies on paratenic hosts showed that *T. canis* larvae penetrate the small intestine walls when ingested and disseminate through the soft tissues of the body via systemic circulation. According to the literature, the most commonly affected organs are the liver, lung and eyes. The central nervous system, heart and skeletal muscles are affected less often [7]. In humans and mice, *T. canis* larvae do not develop to the adult stage but rather migrate throughout the somatic tissue and persist in the infective larvae for extensive periods. In this context, experimental infection in mice, which mimics the biology of human infection, might be relevant to a better understanding of human toxocariasis [8]. There is no report on migratory behaviour and pathology of *T. canis* larvae in mice in India. Thus, the aim of this study was to evaluate the migratory route and histopathology of parasitized tissues during early *T. canis* infection in mice.

2. Materials and Methods

2.1. Parasite

Adult *Toxocara canis* worms were collected from naturally infected puppies kept at Blue cross of India, Tamil Nadu, after deworming with Piperazine hydrate @ 100 mg/kg. The eggs were isolated and cultured according to the method of Fan *et al*. [9] with minor modifications. Briefly, the female worms were dissected at anterior one third of the worm, the uterus was removed by gentle mechanical pressure and the released eggs were collected. The eggs were incubated for 4 weeks in 2% formal saline at room temperature (~26°C) to induce embryo nation.

2.2. Animals

A total of twenty four male Balb/c mice aged 6-8 weeks weighing 15-20 gm were obtained from the laboratory animal house at Madhavaram, TANUVAS. The mice were maintained on commercial pellet feed and *ad libitum* water. They were kept on a normal light/dark cycle (12 h) in a climate-controlled environment (23°C) throughout the study. The mice were maintained and infected in accordance with institutional and national guidelines. The protocol was approved by the Institutional Animal Ethics Committee of TANUVAS, Chennai (Protocol No. 2345/16/DFBS dt.26.10.2016).

2.3. Experimental infection

Experimental infection was performed using embryonated eggs of *T. canis* as per the method described with minor modifications [8]. The *in vitro* embryonated eggs were washed repeatedly in PBS and counted. The mice were divided into 3 groups; Group I: infected with 100 doses; Group II: infected with 1000 doses; Group III: uninfected control mice. The mice were infected with embryonated eggs of *T. canis* by intra-gastric intubation.

2.4. Euthanasia and sample collection

Euthanasia was carried out by cervical dislocation under chloroform anaesthesia at 7, 14, 21 and 28 days post infection (dpi). On each date, two infected mice from each group were sacrificed for larval recovery and histopathology of tissues. The controls were euthanized at the end of the study. Following necropsy, the visceral organs like the lungs, liver, kidneys, brain, eyes and part of carcass muscle were removed. A small piece of any lesions suspected to be due to larval migration was cut from above visceral organs and was fixed in 10% neutral buffered formalin for histopathological examination. The remaining portions of each organ were weighed separately and used for larval recovery.

2.5. Pathological observations

The organs and tissues were analysed macroscopically for the presence of any visible lesions at necropsy. Histopathological examination was carried out at 7, 14 and 28 dpi, the portions of tissue that had been submerged in 10% neutral buffered formalin. The fragments were embedded in paraffin and 4 µm thick sections were cut and stained with Hematoxylin and Eosin (H&E). The pathological changes of the tissues were examined under a light microscope.

2.6. Recovery of *T. canis* larvae in tissues

The organs were weighed, sliced finely with scissors and placed individually in a modified Baermann apparatus in PBS buffer for 4 h at 37°C as per the method of Resende *et al*. [8]. The larvae were found settled at the bottom of the container, which were recovered after centrifugation and removal of supernatant fluid. Subsequently the pellet was fixed with 10% formalin and quantified under a light microscope.

3. Results

3.1. Evaluation of *T. canis* larval migration patterns

Larvae of *T. canis* were recovered from both the groups of mice infected with 100 and 1000 embryonated eggs (Table 1 and 2). In group I mice were infected with the lower dosage (100 eggs), close to natural infection, allowed penetration of low numbers of larvae. In the group I infected mice, maximum larval recovery was from the liver on 7 dpi while after 14 dpi, the majority of larvae were found in the brain and part of carcass muscle. No larvae were found in the lung, kidneys and eyes throughout the experimental period.

Table 1: Mean number of *Toxocara canis* larvae recovered from organs and tissues at various times after experimental infection of Balb/c mice with 100 eggs (Group-I)

Days PI	Liver		Lung		Brain		Kidneys		Carcass muscle		Eyes
	Weight (g)	No. of larvae	Weight (g)	No. of larvae	Weight (g)	No. of larvae	Weight (g)	No. of larvae	Weight (g)	No. of larvae	No. of larvae
7	1.33	4(5,3)	0.28	0	0.28	0	0.49	0	0.92	2 (1,3)	0
14	1.20	2(3,1)	0.26	0	0.26	6(7,5)	0.52	0	0.95	4 (5,3)	0
21	1.23	1(0,2)	0.33	0	0.30	5(4,6)	0.40	0	0.93	4 (2,6)	0
28	1.39	0	0.27	0	0.33	6(8,4)	0.46	0	0.88	5 (6,4)	0

PI-Post Infection

Table 2: Mean number of *Toxocara canis* larvae recovered from organs and tissues at various times after experimental infection of Balb/c mice with 1000 eggs (Group-II)

Days PI	Liver		Lung		Brain		Kidneys		Carcass muscle		Eyes
	Weight (g)	No. of larvae	Weight (g)	No. of larvae	Weight (g)	No. of larvae	Weight (g)	No. of larvae	Weight (g)	No. of larvae	No. of larvae
7	1.21	16 (15,17)	0.28	2 (3,1)	0.23	7 (9,5)	0.42	0	0.86	5 (4,6)	0
14	1.02	12 (9,15)	0.15	1 (0,2)	0.32	11 (10,12)	0.48	0	0.85	8 (9,7)	0
21	1.37	10 (6,4)	0.25	1 (1,1)	0.27	13 (11,15)	0.58	0	0.98	13 (16,10)	0
28	1.29	0	0.32	0	0.30	17 (14,20)	0.56	0	0.94	15 (17,13)	0

PI-Post Infection

In group II infected mice; a maximum number of larvae were obtained in the lung (2 nos) and liver (16 nos) respectively on 7 dpi which decreased thereafter. The larvae were recovered from 7 dpi and on all subsequent dates, with a maximum

number of larvae were seen in the brain (17 nos) and carcass (15 nos) on 28 dpi (Fig. 1ab). There was a significant increase of larval recovery in the brain and part of carcass muscle as the infection progressed. No larvae were found in the kidneys and eyes of all infected mice throughout the study. Moreover, there was a significant increase of larval recovery in the group II in comparison with the group I at all time points post infection.

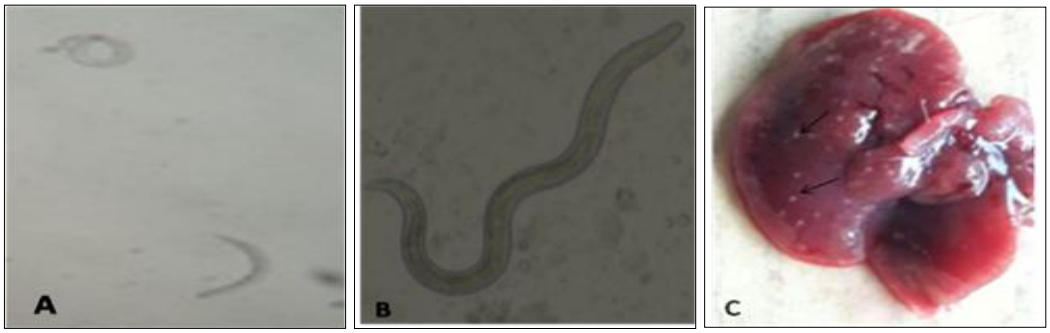

Fig 1: a & b: Migratory larvae of *T. canis* recovered from brain of infected Balb/c mice (10X) and (40X). c: Multiple milky white spot like lesion on the liver of group II infected mice

3.2. Histopathology of infected mice tissues

Macroscopic examination at necropsy revealed enlargement and multiple milky white spot like lesions on the surface of liver of infected mice in group II (Fig. 1c). Histopathological examination of lungs of both the infected groups from 7 to 28 dpi showed congestion, haemorrhage and mild to moderate inflammatory changes on the lung parenchyma with perivascular, peribronchiolar mononuclear cell infiltration predominantly lymphocytes, eosinophils and macrophages (Fig. 2ab). On 28th day, one of group II mice showed diffuse bronchointerstitial pneumonia with predominantly eosinophilic infiltration. Bronchial lumen showed section of larva with granulomatous lesions in the lung parenchyma (Fig. 2c).

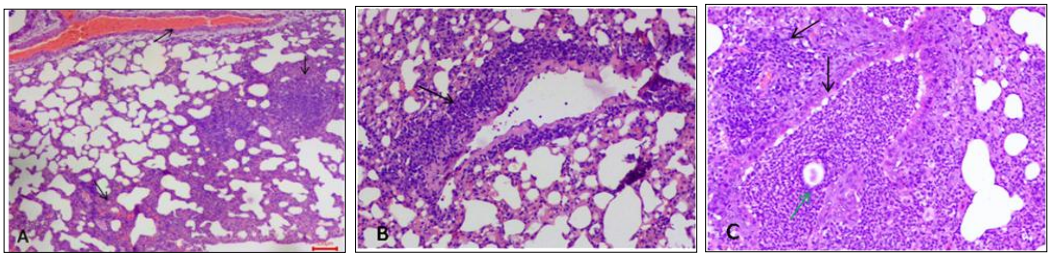

Fig 2: Lung parenchyma of *Toxocara canis* infected Balb/c mice on 28 dpi at inoculation of 1000 EE (H&E). 10X a: Showing pulmonary congestion, haemorrhage with interstitial mononuclear cells b: Eosinophilic infiltration in the alveolar and peribronchiolar regions (Black arrows). c: Presence of granulomatous lesion with trapped larvae in the bronchial lumen (Green arrow). Bar-100µm, dpi: day post infection, EE: Embryonated eggs.

The histological sections of liver of both the infected groups on 7 to 28 dpi showed congestion, mild to moderate inflammatory reaction in the hepatic parenchyma and periportal region (Fig. 3a) mainly eosinophilic infiltration and hydropic degeneration of hepatocytes. On 28 dpi, in both the infected group, typical organized multifocal granulomas were seen in the liver. In the group I infected mice, granulomatous lesions with encapsulated larvae were seen in the liver parenchyma (Fig. 3bc).

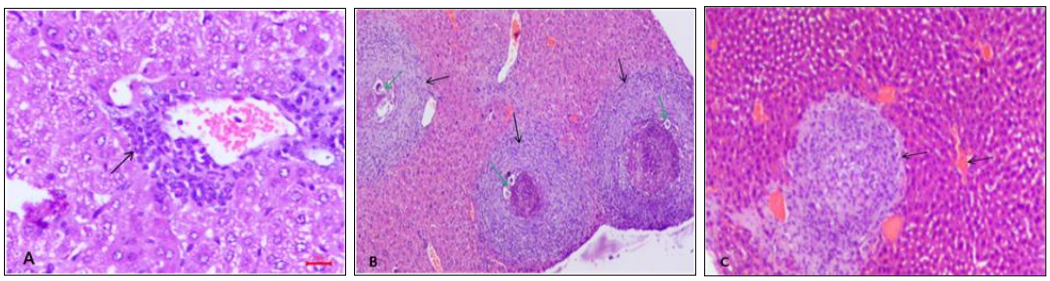

Fig 3: Liver parenchyma of *Toxocara canis* infected Balb/c mice (H&E). a: 7 dpi at 1000 EE, presence of periportal eosinophilic infiltration (20X) (Black arrow). b: 28 dpi at 100 EE infected mice, presence of typical granulomas with encapsulated larvae in the hepatic parenchyma (4X) (Green arrows) c: 28 dpi at 1000 EE infected mice showing granulomatous lesion with haemorrhagic areas (4X) (Black arrow) dpi: day post infection, EE: Embryonated eggs.

The histological sections of the brain as well as eyes of both the groups showed no inflammatory reactions (Fig. 4a) throughout the experimental period. On 28 dpi, in the group II infected mice showed numerous sections of *T. canis* larvae in the cerebrum (Fig. 4b), with fewer larvae detected in the cerebellum (Fig. 4c). Congestion, haemorrhagic areas (Fig. 5a) and eosinophilic inflammatory reaction (Fig. 5b) were seen in the kidneys of both the infected groups on 7 and 28 dpi.

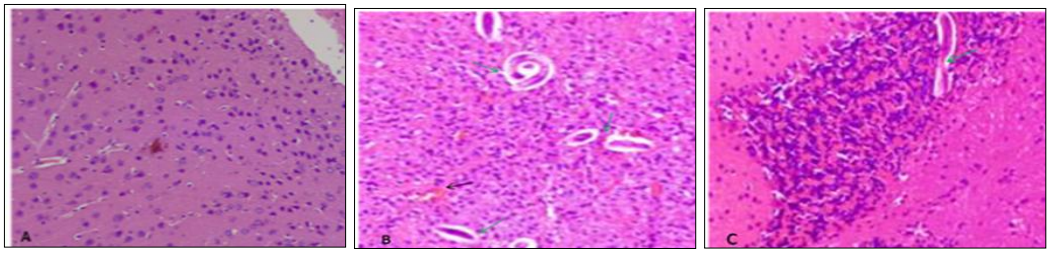

Fig 4: Brain sections of *Toxocara canis* infected Balb/c mice at inoculation of 1000 EE (H&E). a: 7 dpi, no inflammatory changes in the brain tissue (10X) b: 28 dpi, presence of numerous trapped *T. canis* larvae in the cerebrum (Green arrows) (10X) c: showing larva in the cerebellum (10X) (Green arrow) without inflammatory reaction surrounding the larvae. dpi: day post infection, EE: Embryonated eggs

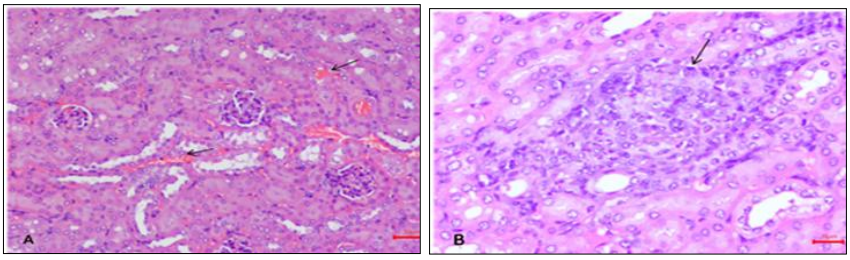

Fig 5: Kidney sections of *Toxocara canis* infected Balb/c mice on 28 dpi at inoculation of 1000 EE. (H&E). a. Showing hemorrhagic areas and congestion (10X). b. Presence of eosinophilic infiltration on the kidney surface (20X) (Black arrow). dpi:day post infection, EE: Embryonated eggs

4. Discussion

The aim of this study was to evaluate the migratory pattern and pathological changes of parasitized tissues, during early *T. canis* infection in mice. The recovery of *T. canis* larvae in mice depends on host strain, dose of inoculated eggs and time post infection [10]. The Balb/c strain is more susceptible to infection by *Toxocara* spp with good larval recovery in the brain and greater survival rate when compared to other strains [10], justifying its use in the present study. Smith [11] reported the similarity of the migratory path in humans and mice and the comparability of the lesions elicited in experimental murine models and humans.

The larval migratory route in the lungs, liver, kidney, eyes, brain and part of carcass muscle of infected Balb/c mice were assessed in this study for 28 dpi. The results coincided with those of Camparoto *et al.* [7] who used a dose of 200 and 1000 embryonated eggs and detected larger number of larvae in the lung and liver after 7 days of infection and maximum amount was found in the brain after 15- dpi in female mice and 30-dpi in males. Different researchers have reported recovery of larvae from the brain and other organs [12-15, 8]. The migratory route of *T. canis* larvae, according to a review by Othman [16] showed that there have been controversies about the migratory route and accumulation of larvae in different organs during the acute and chronic phases. In this study, there was a direct proportional relationship to the parasite load and number of inoculated eggs, as seen by the maximum number of larvae found in the brain of high dose infected groups. The results are contrary to Resende *et al.* [8] who used 1000 embryonated eggs and found that the larvae were recovered from the liver in the first 24 h of infection and peak in the lungs was on the 3 dpi and finally found numerously in the brain after 5 dpi up to 14 dpi.

In our study, the larvae were recovered from carcass muscle of both the infected groups; in absolute numbers, this recovery was directly proportional to infective dose. Similarly, *T. canis* larvae were recovered on 170 dpi from carcasses of all the infected mice at limited doses of eggs [15]. In the present study, no larvae were observed in any of the eyeballs of infected mice at early infection. Whether this is related to the age and quantity of infective eggs used and utilization of different murine strains warrants further investigation. Ophthalmological changes were hard to detect in BALB/c mice and larvae were difficult to identify because of the albino fundi of these mice. Moreover, the corneas of mice had a strong tendency to dry out during observation [17].

Macroscopic examination of visceral organs during necropsy revealed a typical multiple milky white spot like lesions on the liver and kidney. Similar lesions were reported by Al-Saeed and Mahmood [18], who studied the pathological changes in Albino mice infected with different doses of embryonated eggs, of which only 500 eggs infected group showed enlargement of liver with whitish milk spot lesion on the liver surface.

A histopathological study of *T. canis* infected mice over 67 weeks revealed that granulomatous inflammatory lesions were commonly observed in the liver, lungs and musculature from 1 week p.i. onward, but these lesions were rarely seen in the brain. Trapped larvae, however, were found only in histological sections from 12 weeks p.i. or later [9]. In our experiments, the migratory larvae caused inflammatory reaction with haemorrhages in the lungs, liver and kidneys from 7 dpi onwards, but granulomatous lesions and trapped larvae were seen in the liver and lung from 28 dpi. However, in the brain, only numerous trapped larvae were seen without inflammatory reaction in group II infected mice on 28 dpi. Microscopic examination of pulmonary tissues and kidneys showed haemorrhage and inflammatory infiltrate from 7 to 28 dpi of both the infected groups. Similarly, the distributions and pathogenic reactions of *T. canis* larvae in the liver, lung, kidney, heart and brain of Chinese Kun Ming mice were studied after 7-60 days of infection and showed larval fragments and eosinophilic inflammatory infiltrate in all organs except heart from day seven onwards [19]. Mice [20], guinea pigs [21] and monkeys [22] had been evaluated pathologically for use as an experimental animal model for ocular toxocariasis. With these animals, however, the incidence of ocular infection is low and eosinophilic infiltration is rarely observed. Similarly, in the present study there was no ocular pathological changes were observed in infected Balb/c mice.

Our results regarding brain lesions were in agreement with

those of Eid *et al.* [14] showed that, in Balb/c mice infected with *T. canis*, larvae scattered in the parenchyma of the brain with fewer larvae detected in the cerebellum and no visible inflammatory reaction was observed around the migrating larvae. The cerebrum was the preferential location of larvae in the brain and haemorrhages in the brain have also been reported [8]. The reason for the absence of inflammatory cell infiltration in the *Toxocara*-infected brain might be that *T. canis* larvae mimic host tissue antigenic components and escape immune recognition, or perhaps some mechanisms in the nervous tissues operate to diminish inflammation in order to protect themselves from severe injuries caused by inflammation [14].

5. Conclusion

Experimental infection of Balb/c mice with two doses of embryonated eggs of *T. canis* was studied for the first time in India. The migratory larvae induced significant pathological changes in the visceral organs except the brain and eyes of all the infected mice. This was based on the dose of inoculated eggs and period of infection. The results facilitated for better understanding of migration and pathological changes that also might occur during human toxocariasis which may open avenues for future diagnostic and therapeutic options.

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