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Icosiella (Filarioidea) microfilariae from the blood of amphibian hosts: transmission experiments in fish models to detect host specificity

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Abstract. The adult worm of *Icosiella* Seurat, 1917, a nematode parasite has been reported from the connective, sub-cutaneous, inter muscular tissues as well as the intestine and stomach of anuran hosts. Its microfilariae (mf) are the early larval stages, found in the blood of infected hosts. The blood of *Hoplobatrachus tigerinus* Daudin, 1803 (n = 105) and *Bufo melanostictus* Schneider, 1799 (n = 95) was examined for the presence of blood parasites. The blood was infected with sheathed (*H. tigerinus*) and unsheathed (*B. melanostictus*) microfilariae of the nematode, *Icosiella* measuring 59.01 µm in length and 5.46 µm in width. Adult worms were not detected in both species. The prevalence and intensity were 4.76 and 2.0 – 5.0/100 R.B.C.'s in the former and 10.52 and1.0-3.0/100 R.B.C.s respectively in the latter. Cross transmission experiments were performed to test the adaptability of *Icosiella* mf in another cold-blooded vertebrate selecting *Clarias batrachus* as the test model. The experiments indicated that the mf failed to establish themselves in the fish host (Group C) although they appeared at day 6 post inoculation in Group B. *Icosiella* mf are specific for *H. tigerinus* blood and *C. batrachus* cannot act as potential hosts for the mf parasite. This is the first attempt to transmit *Icosiella* mf from amphibian to fish blood.

Key words: *Icosiella,* microfilariae, *Bufo melanostictus, Hoplobatrachus tigerinus, Clarias batrachus,* host specificity.

Introduction

The class Amphibia is a highly diverse class of cold blooded (ectothermic) vertebrates comprising over 8000 species (Wake & Koo 2018) and are important as they have been used extensively in teaching and research. Amphibians have been one of the first group of vertebrate organisms for familiarizing with anatomy and have also served as important experimental models. However, the amphibians are undergoing the most drastic pruning and around half of amphibian species are declining, while a third are already threatened with extinction.

Parasitic infections in amphibians have very often been overlooked although the parasites responsible for amphibian deformities are continuously increasing in numbers (Todd 2007). The parasitic group includes tapeworms, nematodes, flukes, trematodes, filariae, protozoans and fly larvae. Very few parasitic surveys on frogs have been carried out in India, the degree of this haemoparasite diversity remains unknown (Gupta & Khan 2002, Rastogi & Gupta 2003, 2006, Gupta et al. 2006a, 2012, 2014). However, a knowledge on the diversity of these blood parasites is necessary in order to take up further studies to elucidate the effects that these parasites may have on their natural hosts, which may assist in devising suitable protocols for amphibian conservation, now an issue of global concern. The genus *Icosiella* Diesing, 1857 was erected with *Icosiella neglecta* as the type species. The adult worm has not only been reported from the connective sub-cutaneous or inter muscular tissues but has also been recorded from the intestine and stomach of anuran hosts. Bursey et al. (2003) described adult *Icosiella turgeocauda* from the intestinal mesenteries of *Rana cancrivora* from the Republic of Philippines.

Attempts have also been made to observe the effect of blood parasites of *B. melanostictus* on host haematology (Gupta et al. 2006b) and treatment protocols for the control of blood parasites of *Rana tigrina* with the help of the antibiotic, tetracycline (Rastogi & Gupta 2007). Extracellular nematode microfilariae recorded from blood smears obtained from toe-clips were compared with those from the heart from amphibian hosts, *Rana vaillanti* and *Eleutherodactylus fitzingeri* from Costa Rica and whether microfilariae density is correlated with adult filarial worm intensity was also tested (McKenzie & Starks 2008). Netherlands et al. (2015) conducted a multispecies haemoparasite survey by screening the blood from 29 species and 436 individual frogs from three localities in sub-tropical KwaZulu-Natal, a hotspot for frog diversity. Extracellular parasites included trypanosomes and microfilarid nematodes, the latter accounting for only 0.6% of the infectivity. Shannon (2016) examined newly metamorphosed and adult amphibians from 5 families and 9 species for blood parasites from five locations in north central Oklahoma recording the presence of trypanosomes and *Hepatozoon*, but mf was not reported. Nguiffo et al. (2019) examined goliath frog, *Conraua goliath* for microfilarial infection and 42.3% of the frogs were infected.

Microfilarae are the early larval stages of filarial nematodes, found in the blood of infected hosts. During the present studies, the blood of amphibian hosts *Hoplobatrachus tigerinus* and *Bufo melanostictus* was examined to determine microfilarial prevalence. The microfilarial inoculum were injected in a cold-blooded host in order to test their potentiality of survival (if any) in adjacent phylum, Pisces selecting *Clarias batrachus* as the fish model.

Materials and Methods

Host collection: Live *Hoplobatrachus tigerinus* and *Bufo melanostictus* were collected from natural habitats of Bisalpur, Uttar Pradesh, India (Table 1). The former were generally collected with the help of a net due to their highly active movements whereas the latter, by hand or hand net as they could be easily trapped due to their docile nature. They were brought to the laboratory and maintained in the laboratory under amphibious condition with adequate food and water.

Table 1. Details of host collection and *Icosiella* microfilariae collected from amphibian hosts.

Hosts	No. of samples	Locality	Coordinates	ASL (m)	Prevalence	Intensity
H. tigerinus	105	Bisalpur, Pilibhit (U.P.)	Lat.: 28 37 N, Long.: 79 48 E	171 m	4.76	2.0 – 5.0/ 100 RBC's
B. melanostictus	95	Bisalpur, Pilibhit (U.P.)	Lat.: 28 37 N, Long.: 79 48 E	171 m	10.52	1.0-3.0/100 RBC's.

Blood Collection: On the spot diagnosis of blood parasites was done by hanging drop preparation in order to observe the infectivity in a host sample. Live parasites could be observed showing slow wriggling movement which indicated positive infection with *Icosiella* mf. Blood was also examined by microhaematocrit (7000 rpm, duration 7 mins) to confirm the presence of parasites.

Preparation of blood films: Blood was collected from the hind legs and examined for live parasites. Permanent smears were prepared by making thin blood films on clean and grease-free slides by aseptic micro techniques in the laboratory, air-dried, fixed in methanol and stained in Giemsa with buffer (pH 7.2) in the ratio of 1:7 for 40 minutes for best results. They were washed thoroughly in running tap water, and finally in distilled water to remove excess stain, drained and allowed to stand on end to dry. The dried slides were mounted in DPX, screened under oil immersion objective and the images were captured with an attached LEICA camera.

Study of parasite intensity: The average intensity was calculated per 100 erythrocytes, by counting the parasites in 100 RBC under 40X of a microscope by blood cell counter.

Cross Transmission Experiments: In order to determine the host susceptibility of parasites, the method of Khan (1972) with slight modification (Mukherjee & Haldar, 1982) was adopted. Experimental frogs were collected from different sites of Rohilkhand region and maintained in the laboratory under amphibious condition. Donor (*Hoplobatrachus tigerinus*) (Group A) and recipient frogs (*H. tigerinus*) (Group B) frogs, 10 each in the control and experimental group and 10 fish (*Clarias batrachus*) (Group C) were selected for the experiment. It was ensured that the recipient animals were free from parasitic infection by examining them on alternate days for 2 weeks before inoculation. The donor frogs were examined for the infection and the parasites (per 100 RBC) were counted.

Icosiella mf from *H. tigerinus* were inoculated in the fish host, *C. batrachus* and in *H. tigerinus* as follows:

Group A - *H. tigerinus* control (n = 10)
Group B - *H. tigerinus* inoculated with *Icosiella* mf (n = 10)
Group C - *C. batrachus* inoculated with *Icosiella* mf) (n =10)

Group B (*H. tigerinus*) and Group C (*C. batrachus*) hosts were simultaneously inoculated with *Icosiella* mf. Prior to inoculation, it was ensured that the recipient hosts were free from any blood pathogen by examining their blood twice a week for 30 days prior to inoculation. After parasite transfer, the blood of recipient hosts was examined every 24 hours for 21 days to confirm infectivity and intensity.

Inoculum preparation: About two ml. of cardiac blood was removed from the donor host (*Hoplobatrachus tigerinus*) by cardiac puncture. The inoculum was prepared by taking 1 unit (¼ of 1 ml) of peripheral blood in a heparinized syringe containing 1ml. of 0.2% sodium citrate solution from an infected donor. 100 mg of glucose (D-Glucose from Qualigens) was dissolved in 10 ml of saline water (8.5%). 1 unit of salinated glucose solution was mixed with 1 unit of blood in a syringe and the inoculum was injected intraperitoneally into uninfected *H. tigerinus* and *Clarias batrachus*. Each recipient received 0.6ml of the citrated blood. The frogs (cages) and fish (aquaria) were kept under favourable conditions of oxygen, food and temperature. Controls were also maintained throughout the course of experimentation. After inoculation, the blood of recipient frogs and fish were examined after every 24 hrs, for 21 days to detect mf parasites.

Fish host: *Clarias batrachus* collected from the fish market of Bareilly were transported alive to the laboratory. They were maintained in aquaria under proper conditions of food and aeration. A preliminary scan of the blood samples was done to ensure that they were free from any blood infection. Infection free fishes were treated as recipient for inoculation experiments.

Results

Nematode mf belonging to the genus *Icosiella* Seurat, 1917 (Figure 1), identified according to Anderson (2009) were observed in both host species examined, *Hoplobatrachus tigerinus* and *Bufo melanostictus* as per the details provided in Table 1. In the former, they were sheathed, in the latter, unsheathed. Unsheathing is a biological phenomenon in microfilariae and is related to the development of the parasite. It maybe possible that that microfilariare of *Hoplobatrachus* and *Bufo* represent different stages of development.

Group B hosts revealed the presence of mf in blood circulation after a latent period of six days and persisted up to 21 days. The parasites failed to establish in Group C hosts throughout the period of experimentation, despite second inoculation on day 7 (Table 2).

The mf of *Icosiella* were observed in the live condition in fresh blood and also in methanol fixed Giemsa stained permanent smears. Being large in size, the mf sluggishly moved extra-cellularly in between the blood corpuscles. Taxometric identification was made from permanent smears. These mf measured 59.01µm in length and 5.46µm in width. The body was elongated and enclosed in a sheath (Fig. 1 A-C) in *H. tigerinus*. As the microfilariae matured (Fig. 1B), the sheath gradually extended and finally it could be seen to cast off its sheath (Fig. 1C). However, in *B. melanostictus,* unsheathed microfilariae were observed (Fig. 1D). In the former, the sheath was longer than the body and did not take a deep stain as compared to the cell body. Width of head level was 7µm, at the anterior tip of sheath 24µm, length of extension from head region to tip was 6µm. At the tail end, the maximum width was 8µm and from the tail end to the posterior end it was 12µm.

The mf took a deep stain. Cephalic space was clearly demarcated measuring 44.06 percent of body length. The tail space was 5.08 percent of body length. The posterior end tapers abruptly and is blunt in shape. Fine annulations could be seen over the whole body of the nematode by phase contrast microscopy.

Discussion

The objectives of present study were to examine the blood of amphibian hosts, *Hoplobatrachus tigerinus* and *Bufo melanostictus* in order to determine microfilarial prevalence. The microfilarial inoculum were further injected in *Clarias*

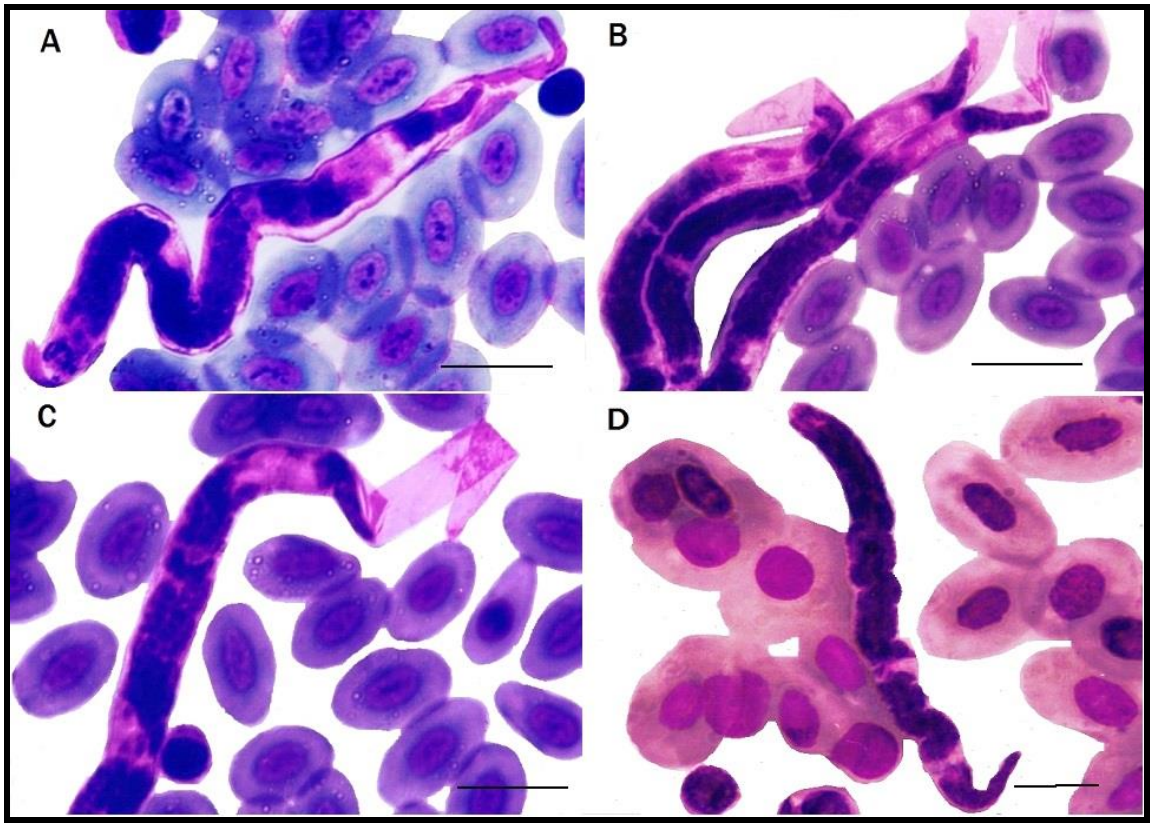

Figure 1. *Icosiella* microfilariae from amphibian hosts. A-C Sheathed larva *from Hoplobatrachus tigerinus* (A Young microfilaria, B Growing microfilariae, C Mature microfilaria shedding its sheath). D Unsheathed microfilaria of *Bufo melanostictus* (Bar indicates 10 microns).

Table 2. Cross transmission experiments of *Icosiella* microfilariae (2.0-5.0/100 R.B.C.'s) (donor *H. tigerinus*) in *C. batrachus* (0.6ml inoculum/host).

Recipient Host	Group (n=10)	Duration (days)																					Intensity (/100 R.B.C.'s) after 21 days
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	
H. tigerinus	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Nil
H. tigerinus	B	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1.5-2.5
C. batrachus	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Nil

batrachus in order to test their potentiality of survival in the selected fish host. During the present course of investigations, *Icosiella* mf were observed in the blood of amphibian hosts, *H. tigerinus* and *B. melanostictus*. There are no records of the parasite or its larvae from the area chosen for investigation, the Rohilkhand region or its vicinity and records of the parasite from the Indian subcontinent are meagre.

Icosiella has been described from 4 genera of frogs *Rana, Discoglossus*, *Babina* and *Cornufer* (Purnomo & Bangs 1996). *I. intani* mf are significantly larger, more slender and sheathed. The present parasite from *H. tigerinus* is also quite large and is sheathed. However, it does not exist as slender forms as reported for *I. intani,* on the contrary, it is stout and broad.

Twenty-three species of haemoparasites from eleven amphibian and reptiles species were recorded in Ryukyu Island, Japan (Miyata et al. 1978). Out of these, 2 species of mf were detected: one from *R. holsti* (99.0-120.0µm long, 3µm wide), as the adult worm was not obtained therefore, the mf were not identified; the other mf was identified as *I. sasai* Hayashi, 1960 from *R. subaspera* (79.8-94.8µm long) which was apparently different from the former in shape and size.

Microfilariae were observed by Barta & Desser (1984) from *B. americanus* (120 x 1.6µm) and another smaller species in *R. clamitans, R. sylvatica* and *R. septentrionalis* measuring 73.8 µm (65.6 µm -82.0 µm) by 1.2µm (35). Werner (1993) examined blood parasites from 246 amphibians. Microfilariae of *I. neglecta* were seen in 5 *R. nigromaculata* and the author opined it could be the same mf as illustrated by Miyata et al. (1978) from Ranids in Japan. Due to lack of availability of the adult nematodes from the ranid hosts, it is difficult to make references on the taxonomical status of the mf discovered from the blood of *H. tigerinus*. However, from the available literature on the mf of nematodes discovered from amphibians, the present mf resembles those recorded by Werner (1993) from the blood *R. nigromaculata* which was identified by the author as *Icosiella neglecta* Diesing, 1851.

With increasing emphasis on immunological aspects of parasitism, investigators are faced with the problem of obtaining parasites and the larval stages in large numbers, free of extraneous material to extract and characterize functional and nonfunctional antigens. Keeping the above

facts in view, these preliminary studies on *Icosiella* may provide incentive in utilizing these parasites for further studies on host parasite density dependent mechanisms and as valuable tools for immunological studies. *Bufo* blood samples also revealed *Icosiella* mf. The present mf differs from that of *I. intani* and also from *H. tigerinus* mf in lacking a sheath. However, the other structural features are similar to those of the microfilariae from *H. tigerinus*. It may be possible that both the mf from *H. tigerinus* and *B. melansotictus* are transmitted by the same or similar mosquito species, the differences being in the sheathing/exsheathing of microfilariae. However, further studies on larval development and transmission of *Icosiella* in the vector are required before any safe conclusion in this regard may be made.

Parasite transfer experiment

Our attempts to establish the parasites in related/unrelated hosts were unsuccessful. Hysek & Zizka (1976) could successfully transmit the parasite from cold blooded to warm blooded animals. However, the parasitic forms resembled the forms from tadpoles and young frogs and not from the adult frogs contrarily, all other workers failed to establish the parasite in unrelated hosts.

During the present studies, *Icosiella* mf appeared in peripheral circulation on day 7 p.i. (Group B) and persisted with an intensity of 1.5 to 2.5/100 R.B.C's, Group C hosts were devoid of any infection. The experiment conducted suggests the incompatibility of *Icosiella* to survive in alternate hosts. It is also evidenced that the parasite could establish in their natural hosts under experimental condition of parasite inoculation although their intensity in such cases was lower as compared to natural infections. It appears that the haemoparasites are not adaptive to divergent conditions of microenvironment as *Icosiella* which is a natural parasite of amphibian hosts failed to establish in fish host although the latter is also a cold-blooded vertebrate.

It has been postulated that the mutual adaptation of host and parasite becomes manifest as a strict specificity and usually develops during the course of prolonged period of close association between phylogeny of parasite and hosts (Dogiel 1964). The capacity for changing hosts might depend on the range of reactions of the parasite to the hosts and if the physiological and morphological mechanisms of the parasite are flexible enough to permit the parasite to overcome the resistance of the new host, then the parasite might be successful in adapting itself in the new host environment. New host transfer is also determined by the degree of natural immunity of the parasite and the factors which help to overcome it. Shulman (1958) emphasized the importance of duration of association between the parasite and hosts has been emphasized suggesting that host change becomes difficult in older host parasite associations. This would implicate that *Icosiella* microfilariae have an old association with *H. tigerinus* rendering them difficult to survive in other cold-blooded hosts.

Specificity is regarded as a characteristic feature of parasitism and for its proper elucidation several factors have to be taken into consideration. Period of contact, geographical distribution, intraspecific variations, transmission, developmental cycle, pathological and physiological effects, ecological factors affecting epizootiology, resistance and immunity have been regarded as important variables in determining the specificity of parasite which have to be taken into account for assessing all host-parasite relationships.

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