Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.elsevier.com/locate/apjtb

Document heading doi:10.1016/S2221-1691(12)60068-9 © 2012 by the Asian Pacific Journal of Tropical Biomedicine. All rights reserved.

Incidence and histopathology of encysted progenetic metacercariae of *Clinostomum complanatum* (Digenea: Clinostomidae) in *Channa punctatus* and its development in experimental host

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ARTICLE INFO

Article history:

Received 18 October 2011

Received in revised form 12 November 2011

Accepted 8 December 2011

Available online 28 June 2012

Keywords:

Clinostomum complanatum

Encysted progenetic metacercaria

Channa punctatus

Histopathology

Experimental infection

Ovigerous adult worm

Incidence

Host–parasite interaction

Metacercarial transformation

ABSTRACT

Objective: To study the incidence of encysted progenetic metacercariae of *Clinostomum complanatum* (*C. complanatum*) in *Channa punctatus* (*C. punctatus*), associated histopathology and the experimental infection to laboratory chicken to obtain ovigerous adult worms. **Methods:** Live *C. punctatus* were brought from local fish market of Aligarh, India, dissected and examined on a monthly basis for the presence of *C. complanatum* cysts. For histochemistry, infected tissue sections with attached cysts were processed for haematoxyline and eosin staining. Cysts were aseptically fed to 4 day old leghorn chicken to obtain adult worms. Mechanically excysted metacercaria and the ovigerous adult worms were stained in carmine to prepare permanent slides. **Results:** One year survey for the infection of encysted progenetic metacercaria of *C. complanatum* in *C. punctatus* revealed the prevalence, intensity and abundance of 24.7%, 2.27 and 0.608, respectively. Histopathology showed heavy infiltration of immune cells at the site of cyst attachment and some tissue damage was also evident. Following feeding to experimental chicken, about 41.07% of the encysted metacercariae were able to excyst and migrate back to bucco-pharyngeal region where they tenaciously attached and fed on blood, and transformed into ovigerous adult worms from 62 hours onwards of post infection. **Conclusions:** The parasite is potentially pathogenic to the host, and the availability of a suitable intermediate host can be a contributing factor for the occurrence of *C. complanatum* metacercaria either in the excysted or encysted form, indicating loose host specificity and zoonotic potential.

1. Introduction

Fishes are good source of quality protein, but various diseases including parasitic infections pose a threat to fish culture[1]. In addition to the economic loss to farmers, many of the parasites, particularly trematodes, are also of zoonotic importance. Eating raw or, improperly cooked or processed fish is the main source of these infections to human that has been reported from various geographical regions[2], causing laryngitis. The World Health Organization (WHO) has estimated that the number of people currently infected with fish-borne trematodes exceeds 18 million, and many more are

at risk[3].

Clinostomum complanatum (*C. complanatum*) (Rudolphi, 1814), a common clinostomid trematode found in the esophagus of ardeiid birds is widely distributed across the world, and in India the infective larval stage of this parasite is usually found in the body cavity of forage fish, *Trichogaster fasciatus* (*T. fasciatus*) (syn: *Colisa fasciata*), as non-encysted metacercariae with an infection rate of 98%–100%[4]. However, during our recent survey, it was observed that the encysted form of the progenetic metacercariae of this species was also found in the economically important food fish, *Channa punctatus* (*C. punctatus*) (Bloch, 1793), which serves as the second intermediate host for this parasite. The metacercariae of *C. complanatum* were known to cause considerable damage to the viscera and musculature of many fish species[5], however, the damage exerted to *C. punctatus* is not known.

Abidi and Nizami[6] successfully established the

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Foundation Project: This work was financially supported by Aligarh Muslim University (grant No. 09PHDL128).

experimental infection of non-encysted metacercaria of *C. complanatum* from *T. fasciatus* in chicken, but there is no report of such studies on the encysted form of this parasite from *C. punctatus*. In the present study, a survey was carried out for one year to assess the incidence of infection in *C. punctatus* and the encysted progenetic metacercariae of *C. complanatum* obtained from the infected fish were fed to experimental leghorn chicken and their maturation into ovigerous adult stage was studied.

2. Materials and methods

2.1. Epidemiological study

Live *C. punctatus* of 7–16 cm long size were brought in pond water from local fish market of Aligarh (latitude: 27°, 54' N; longitude: 78°, 05'E), U.P., India, to the lab on a monthly basis and maintained in aquaria until used. Within three days, these fishes were observed externally for the presence of metacercarial cysts, commonly known as yellow grub. The dissected fishes were carefully observed for the presence of encysted progenetic metacercariae of *C. complanatum* in all the internal organs. The attached cysts visible to the naked eye were photographed and then removed and transferred into 0.75% normal saline and their number was recorded systematically. The cyst wall was teased carefully using forceps to liberate the metacercaria which was fixed in 70% alcohol and permanent slides were prepared after staining in acid carmine.

2.2. Histopathological study

Some cysts with attached tissue of the infected fish were immediately washed and fixed in 10% neutral buffered formalin, processed further and embedded in paraffin wax to cut 7 μ m thick sections using a rotatory microtome, stained with haematoxylin and eosin to study histology and cellular infiltration at the site of attachment. The DPX mounted sections were observed under microscope and photographed.

2.3. Experimental infection to chicken

Properly washed 4 cysts were carefully removed from the infected fishes and fed to each of the 14 four-day-old leghorn chicken using a soft brush, which were maintained in separate cages and provided commercially available chicken food and water *ad libitum*. The buccal cavity of each chick was regularly observed to detect the appearance and attachment of the parasite and they were necropsied daily until seven days post infection (p.i.). Developmental stages and adult worms obtained from chicken were stained in acid carmine to prepare permanent slides. The voucher specimens were submitted in the Museum of the Zoology Department, A.M.U. Aligarh.

3. Results

3.1. Epidemiology

The body surface of the fish *C. punctatus* appeared normal and no cyst was found to be attached on it. Following necropsy, the infection was largely found at the dorso-lateral sides of pre-opercular region either as an isolated or clumps of cysts (Figure 1). These cysts were visible with naked eyes (2–3 mm) and appeared yellow, light yellow or white colour. In addition, three cysts on the gill and eight on the kidneys were also found attached in different fishes. From the microscopic studies of mechanically excysted metacercaria, it is reported for the first time that the progenetic metacercaria is *C. complanatum*, which was found in the encysted form in the intermediate host, *C. punctatus* (Figure 2), unlike the excysted form occurring in *T. fasciatus*. Interestingly, the size of the host fish had no correlation with the infection rate, but the size of the worms was comparatively bigger than that of non-encysted metacercaria and showed active movement once they were mechanically excysted. The morphological features corresponded well with that of non-encysted metacercaria from *T. fasciatus*, which were characterized by the presence of bifurcated gut extending to posterior most region of the worm, quiescent gonads, and narrow streak like uterine tube extending posterior to acetabulum and the position of gonads, etc.

Figure 1. The body cavity of *C. punctatus* showing encysted progenetic metacercaria of *C. complanatum* (arrows).

In the present study, the prevalence of encysted progenetic metacercariae of *C. complanatum* infection in *C. punctatus* was quite varied from month to month. It was maximum in December (52.6%), followed by January (48.1%), while infection was not detected (0%) during October and minimum infection was observed in September (7.6%). The range of cysts varied between 1 and 12 per infected fish. The mean intensity was maximum in July, whereas the highest

abundance was in January (Table 1 and Figure 3).

Figure 2. The encysted progenetic metacercaria of *C. complanatum* (PM) and four day old ovigerous adult worm (OAW) obtained from experimentally infected chicken.

Eggs can be seen in the uterine sac (scale 1.04 mm).

Figure 3. Seasonal variation of encysted progenetic metacercaria of *C. complanatum* infection in *C. punctatus* in Aligarh region of north India during April 2010–March 2011.

3.2. Histopathology

The histochemistry of the infected host tissue revealed heavy infiltration of lymphocytes and other immune cells all around the cyst wall and adjoining tissues (Figure 4, 5, 6), while the enclosed progenetic metacercaria appeared free from the cyst wall. The tissue layer surrounding the cyst wall

was fibrotic and slightly necrotic, whereas the underlying tissue appeared normal (Figure 7). The attached muscle and kidney (Figure 8) appeared necrotic at the host–parasite interface, probably due to inflammatory reactions, clearly indicating that the infection is potentially damaging the infected tissue of the host.

Figure 4. Cross section of the encysted *C. complanatum* metacercaria surrounded by the cyst wall (scale 310 μ m).

Figure 5. The infected tissue engulfed by the sucker of *C. complanatum* metacercaria and the heavy infiltration of immune cells at the site of attachment (scale 260 μ m).

3.3. Establishment of experimental infection

The effort to establish *C. complanatum* infection in leghorn chicken as an experimental definitive host was successful. The worms started appearing in the buccal cavity beneath and/or around the tongue region (Figure 9) after 9 hours of p.i. onwards. The infected chicken were found little

disturbed in their feeding and behavior than control ones. During transformation of progenetic metacercariae into adult ovigerous stage, there was a general increase in worm body size, tremendous colour change of gut contents from pale yellow to dark grey or red, probably due to the ingestion of host blood, enlargement and development of gonads and vitellaria, and the uterus appearing as distended sac full of eggs (Figure 2–OAW). The microscopic observation of the prepared permanent slides of developmental stages recovered from the infected chicken showed that the formation of eggs started from 62 hours of p.i. and the uterus was full of eggs from 72 hours onwards. The worms disappeared on seven days p.i. leaving a scar at the site of attachment. The infection rate in chicken was about 85.7% and a recovery of 41.07% (Table 2).

Figure 6. Heavy infiltration of immune cells in the infected muscle tissue (scale 150 μ m).

Figure 7. The metacercaria encysted on muscle. The infected tissue is necrotic and fibrotic, while the underlying tissue appear normal (scale 350 μ m).

Figure 8. *C. complanatum* metacercaria encysted on the kidney. Note the infiltration, inflammation and tissue damage. Metacercaria (arrow head), cyst wall (arrow), tissue damage (asterisk) and normal tissue (star) (scale 450 μ m).

Figure 9. The buccal cavity of experimentally infected chick showing the attached ovigerous adult worm of *C. complanatum* (arrow).

Table 1The incidence of encysted progenetic metacercariae of *C. complanatum* in *C. punctatus* during April 2010–March 2011.

Months	No. of fishes examined	No. of fishes infected	No. of cysts recovered	Prevalence (%)	Range	Mean intensity	Abundance
April	24	4	11	16.6	1–6	2.75	0.458
May	71	10	35	14.0	1–9	3.50	0.492
June	25	8	19	32.0	1–6	2.37	0.760
July	25	7	28	28.0	1–12	4.00	1.120
August	24	6	10	25.0	1–2	1.66	0.416
September	26	2	3	7.6	1–2	1.50	0.115
October	41	0	0	0.0	0–0	0.00	0.000
November	26	5	10	19.2	1–4	2.00	0.384
December	19	10	20	52.6	1–5	2.00	1.052
January	27	13	31	48.1	1–6	2.38	1.148
February	28	8	18	28.5	1–4	2.25	0.642
March	23	7	20	30.4	1–5	2.85	0.714
Total	364	80	205	24.7	1–5.08	2.27	0.608

Table 2Summary of experimental infection of encysted progenetic metacercariae of *C. complanatum* from *C. punctatus* in leghorn chicken.

No. of chicken used	No. of cysts fed	No. of chicken infected	% of infection	Total no. of ovigerous worms recovered	% Recovery	Worm recovery/chick
14	56 (4 cysts/chick)	12	85.70	23	41.07	1–3

4. Discussion

The infection and infestation of parasites particularly trematodes including *C. complanatum* should be managed for successful aquaculture both from economic and zoonotic point of view. The infection affects the feeding habit, acquisition of body weight and reduced fecundity and even death of the host fish[7]. On the other hand, many reports show that they have zoonotic potential as well. WHO has identified fish-borne trematodes as a serious zoonotic and health concern in one of its reports[3] and Chai *et al*[8] reviewed the same aspect in detail. Many cases of human infection of this parasite have been reported from various regions of the world, and *C. punctatus* is a delicious food fish which served as intermediate host for this parasite, therefore it is important to control this infection to check the possibility of zoonoses.

The adult members of the digenean family Clinostomidae (Luhe, 1901) are parasites of buccal cavity, oesophagus or intestine of birds, and *Clinostomum* (Leidy, 1856) is the major genus of this family. About 54 species have been reported by various authors, even though its classification is quiet controversial and *C. complanatum* is the most frequent species of this genus found in India. At least 24 fish species as the second intermediate host for the metacercarial stage of this parasite have been reported from different parts of the world[9–16], but in India, it is normally found in *T. fasciatus* as non encysted metacercaria[4], however, in the present study we describe the incidence of the same parasite in an economically important food fish *C. punctatus* as encysted metacercaria. Nevertheless, this parasite has been reported in the same host fish (single worm) from Meghalaya, India, with a prevalence rate of 0.74%, but was in non-encysted form[17]. In contrast, we found the encysted form in *C. punctatus* and the rate of incidence was 24.7%. The higher

incidence during the winter months may be due to the higher infectivity of the parasite or increased acquisition of infection during post monsoon and pre-winter months probably due to abundance of snails during this period. The clean and less polluted post monsoon aquatic environment may also promote higher hatching rate of eggs, better survivability and infectivity of the emerged miracidia and cercaria. Similarly, the low incidence during the months following heavy rain and flood in north India in July–August, may be due to the less chances of infection as the concentration of infective larval stages in large volume of water would be low and moreover, whatever miracidia and cercariae were readily available in the habitat might have been washed away along with either rain or flood water, thus suggesting that the macroenvironmental factors could be playing an important role in the abundance of field infections of this parasite.

The results of the present study are in agreement with the previous reports that this parasite caused considerable damage to the viscera and musculature of many fish species[5,18]. The engulfing of host tissue by the sucker, tissue destruction and immune cell infiltration in the infected parts are clearly evident. Recruitment of leucocytes to the site of infection or inflammatory foci suggests a role for fish lymphocytes in the regulation of early immune responses to infection[19], which needs further study to confirm this assumption. In addition, the cysteine proteases in the excretory/secretory products of *C. complanatum* actively degraded a wide variety of host proteins[20] as a survival strategy. It is interesting to note that within the same geographical region but in two different piscine intermediate hosts, the infective larval form of *C. complanatum* was found in excysted form in *T. fasciatus* and in encysted form in *C. punctatus* probably in response to respective host's microenvironment reflecting enormous adaptive survival

strategy adopted by the parasite.

The generation of adult worms from larvae in experimental models is always interesting for comparative biochemistry, physiology and gene expressions in different host environments. During the transformation process from metacercaria to ovigerous adult worm in 4-day-old laboratory chicken accompany a series of changes that are triggered by the factors provided by the experimental host. In the present study, the encysted metacercariae from *C. punctatus* were successfully transformed into ovigerous adult stage in experimental chicken as also reported earlier for the excysted form[6]. The metacercarial cysts fed to the chicken reach the crop where they are excysted and then migrated up to the buccopharyngeal region where they attached to the buccal epithelial layer, actively fed on host blood and transformed into adult ovigerous state. It was also interesting that the two different microenvironments of the hosts force the parasite to adopt different (excysted or encysted) survival strategies, reflecting enormous adaptive features of these organisms.

As baseline information, the present study offers a promising laboratory model to carry out further studies on the pathology, host parasite interaction, immune evasion, biochemical and metabolic switching mechanism, stage specific gene expression and ultrastructural changes during metacercarial transformation into adult worm.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

The authors are thankful to the Chairman, Department of Zoology for providing necessary laboratory facilities and, Professor Mohammad Hayat and Dr. Farman ur Rehman Khan for extending the microscopy facilities. The research fellowship provided by the Aligarh Muslim University is also highly acknowledged.

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