



HISTOPATHOLOGICAL EVALUATION OF *IN VITRO*
ICHTHYOPHTHIRIUS MULTIFILIIS CULTURE IN
RED KOI (AKA MATSUBA) TISSUE

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Summary

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In order to develop any effective means of prevention or therapy against *Ichthyophthirius multifiliis* infection, in this project, the *in vitro* culture of *Ichthyophthirius multifiliis* trophont containing tissue cysts has been developed. This enables researchers to produce antigens and vaccine and to supply pure organisms without any contamination. Healthy Red koi (Aka Matsuba) tail fin and skin were inoculated into tissue culture media, Leibovitz L-15. The trophonts were exposed to tissue culture within 1 hr after removal from the heavily infected Red koi mucus. Twenty-four hours after introduction of trophonts into tissue (skin) culture, most trophonts observed were adjacent to the basement membrane of the epithelial layer of skin. Additionally, higher numbers of mucous cells in the tail fin epithelium were noted 12 h after trophont introduction into culture. The histological sections of the skin of Red koi (Aka Matsuba) revealed large trophonts of the *I. multifiliis* after 10 days that were prominently lodged in the epidermal layers. In this research, the ability to culture *I. multifiliis* opens new opportunities to develop vaccines, test drugs, and clone parasites for genome sequencing.

Key words: fish, histopathological evaluation, *Ichthyophthirius multifiliis*, tissue culture

The freshwater ciliate *Ichthyophthirius multifiliis* causes Ich or white spot disease which is recognised to be one of the most pathogenic diseases of wild and cultured freshwater fish, with significant economic losses to aquaculture industry worldwide (Aihua & Buchmann, 2001). Infections have been reported from all regions where

fish are cultured as well as in most continents (Nigrelli *et al.*, 1976). The three stages in life cycle of the parasite are: an infective theront, a parasitic trophont and a reproductive tomont (Maki & Dickerson, 2003). Mortalities result when large numbers of the mature parasitic trophont stage exit the host causing disruption to

the epidermis and interfere with the osmotic balance of the host. Current strategies for its treatment and control depend on the use of chemical agents such as formalin, chloramine-T and a number of disinfectants that include the active agent's hydrogen peroxide and sodium percarbonate to kill waterborne stages of the parasites. Chemotherapy could however hardly control this parasite after penetration into fish skin and gills. The high cost involved in therapy and the public concern for food and environmental safety are other disadvantages of chemotherapy. Thus, vaccination against *I. multifiliis* can be considered as an alternative to chemical treatments and to prevent mortality in fishes (Maki & Dickerson, 2003). Gamma irradiated (as radiovaccine), formalin inactivated trophont of *I. multifiliis* in combination with alginic acid nano particles were used to immunise the rainbow trout. (Heidarieh *et al.*, 2015).

With *in vitro* culture of organisms it might be possible to produce high quantities of the parasite not only for the purpose of vaccine production but also for other studies, including development of diagnostic tests. Currently fish are used to cultivate the parasite.

There are a number of issues involved in the culture of protozoan parasites, such as *I. multifiliis*, that make these procedures highly complex and subject to many variables, some of which are known (Nielsen & Buchmann, 2000; Xu *et al.*, 2002). *In vitro* culture of organisms at any of these stages within the life cycle involves a tremendous number of variables, including parasite stage, host site, host temperature, host immune responses, parasite species and/or strain, and parasite-protective mechanisms (Diamond, 1987; Evans, 1987; Visvesvara, 1999). The present study was conducted on adaptation

and histopathology evaluation of the *I. multifiliis* to skin culture *in vitro*.

Fish and parasite (Ichthyophthirius multifiliis). Twenty healthy Red koi carps (Aka Matsuba) weighing 4.07 ± 0.63 g from a fish shop in Karaj, Iran were kept in running water (flow rate 1.5 L/min) in polypropylene tanks (38 L) with water temperature 23 ± 2 °C, pH 7.8–8, dissolved oxygen 6.2–8.1 ppm, and natural photoperiod (10L:14D).

The parasite, *I. multifiliis*, was obtained from heavily infected Red koi (Aka Matsuba) caught from a local fish shop. Mature trophonts were collected and transferred to glass aquaria containing aerated tap water at 17–18 °C until all individuals were heavily infected by the ciliate parasite. After 72 h the trophonts in infected fish were collected and counted to prepare vials containing 10 trophonts in each vial (Heidarieh *et al.*, 2014).

Tissue preparation. Healthy Red koi (Aka Matsuba) were anaesthetised in MS-222 (ethyl-m-aminobenzoate, methanesulfonate salt 80 mg/L) and killed by cervical dislocation. Tail fin and skin (from the lateral body wall with sterile instruments into 10.5×7.4 cm pieces) were cut off and placed in petri dishes with sterile tap water. Then, these were rinsed with Penstrep (100 IU/mL penicillin and 100 µg/mL streptomycin; Gibco) and transferred to fresh sterile tap water (10 °C).

Culture media. Tissue culture was performed using the method of Nielsen & Buchmann (2000) with some modifications. Prepared tail fin and skin were washed with Hank's balanced salt solution (Sigma) and were inoculated into tissue culture media, Leibovitz L-15 (Gibco) in a fresh tissue culture flask. L-15 culture media was supplemented with 5% foetal calf serum (FCS), penicillin (100 IU/mL) and streptomycin (100 µg/mL). Cell cul-

ture flasks were allowed to develop skin and tail fin for 2 to 3 days prior to experiments. Live trophonts (20–25 trophonts per tissue culture flask, 25 cm²) were incubated at 25 °C. The trophonts were used within 1 h after removal from the heavily infected Red koi mucus.

Examination of viable theronts and trophonts. The survival and multiplication of theront and trophont in tissue culture were recorded daily for 10 days with an inverse microscope (Labomed TCM-400, America) (magnification 40–100×).

Histological observations. In order to investigate parasite proliferation in tail fin and skin, experimental histopathology study was performed. Tissue culture flasks were emptied of medium by inversion and also aspiration into a solution of bleach. Tissues were fixed by adding 10% formalin buffer solution for 48 h, dehydrated in alcohols and cleared xylene and then embedded in paraffin. Sectioning at a 4–5 µm was done on a rotary rocker microtome, then rehydrated in alcohols and stained with haematoxylin-eosin.

At first, multiplication of organisms was determined by inverse microscopic observation of living cultures. Twenty-four hours after parasite introduction into cultures, most trophonts observed were adjacent to the basement membrane of the epithelial layer of skin (Fig. 1). Additionally, light microscopy of fish tail fin revealed normal appearance (Fig. 2), but higher numbers of mucous cells in the tail fin epithelium were noted 12 h after trophont introduction into culture media. The histological sections of the skin of Red koi (Aka Matsuba) revealed large trophonts of the *I. multifiliis* that were prominently lodged in the epidermal layers after 10 days (Fig. 3). *In vitro* culture of organisms at any one of these stages within the life cycle involves a tremendous number of variables, including parasite stage, host site, host temperature, host immune responses, parasite species and/or strain, and parasite-protective mechanisms (Diamond, 1987; Evans, 1987; Visvesvara, 1999). At the present time, there are advantages in the *in vitro* culture of protozoan parasites;

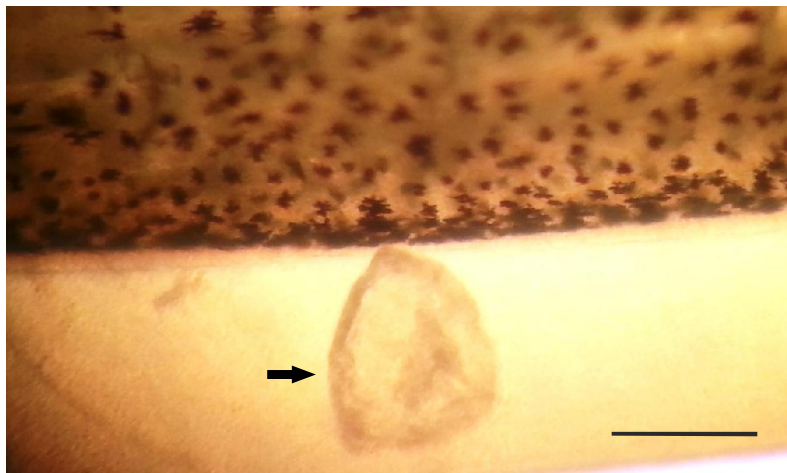


Fig. 1. Trophonts adjacent to the basement membrane of the epithelial layer of fish skin culture after 24 h (light microscope, bar=40 µm).

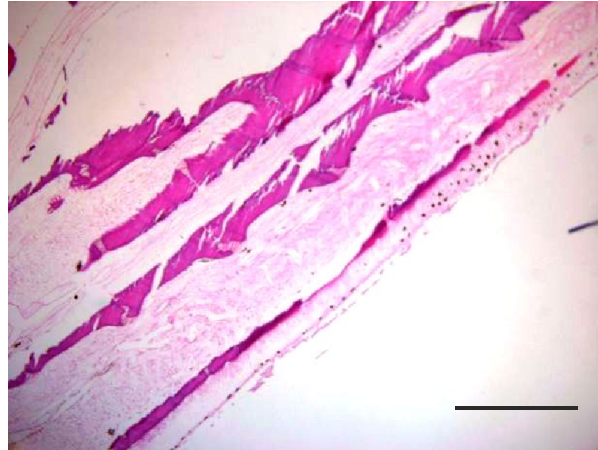


Fig. 2. Fish tail fin culture without any damages (H & E, bar=200 μm).

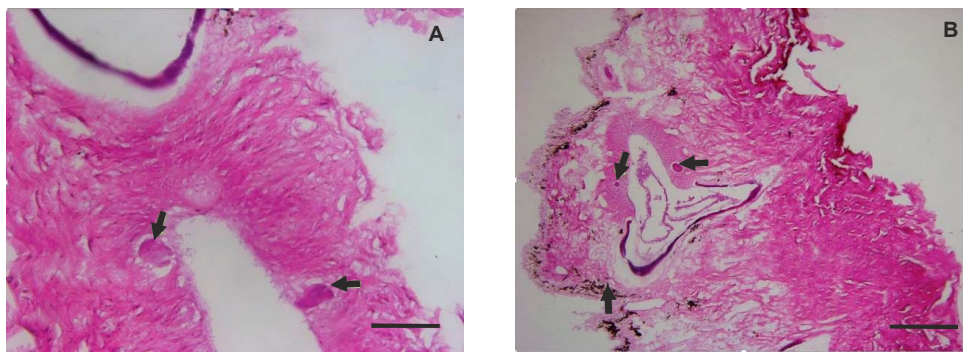


Fig. 3. Parasites appearing in fish skin culture (arrows) after 10 days. H & E, bar=200 μm (Fig. 3A); bar=100 μm (Fig. 3B).

one of the great advantages of *in vitro* culture is that obtaining a continuous supply of pure organisms without any contamination is possible (Trager & Jensen, 1976; Evans *et al.*, 1999). Also, *in vitro* cultivation is important for producing antigens, assessing functional antibodies and cell-mediated protective systems against the parasites, screening drugs, identifying potential therapeutic agents and producing vaccines, as relatively large numbers of parasites at specific stages can be produced in culture.

In summary, results demonstrated current culture conditions suitable for *I. multifiliis* generation *in vitro*. We hope this *in vitro* method will provide a basic research tool for understanding of parasite biochemistry, immunology, biology, physiology and for finding suitable drugs for treatment.

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