Assiut Vet. Med. J. Vol. 41, No. 81, April 1999

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IMMUNIZATION TRIALS AGAINST *ICHTHIOPHTHIRIUS MULTIFILIIS* FOUQUET (CILIOPHORA) IN FISH

(With 2 Tables and 2 Figures)

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(Received at 23/3/1999)

محاولات لإحداث مناعة في الأسماك ضد طفيل "أكثيوفثريس مالتيفلس"

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لحماية اسماك المفرخات من عدوى "أكثيوفثريس مالتيفلس" فقد جرت عدة محاولات لزيادة كفاءة الجهاز المناعي في الأسماك الصغيرة لمقاومة هذا الطفيل والحد من نسبة النفوق في حالة الاصابه به. ومن اجل تحقيق هذا الهدف فقد تم تحضير لقاحين من الطفيل. الأول عن طريق الطحن الدقيق للطفيل والثاني بواسطة إضعاف الطور النشيط بالمعالجة الحرارية. وقد تم استعمال ثلاث طرق مختلفة للتحصين. الأول عن طريق الحقن البريتوني والثاني بخدش الجلد والثالث بعمل حمام مائي تمرر به الأسماك. وقد تم دراسة فعالية كلا اللقاحين بمقدار الحماية الحادثة بعد اختبار التحدي على أسماك المبروك الشعبى. جاءت النتائج مشجعه وكانت نسبة الحماية التي أحدثها اللقاح الأول ١٠٠% و٧٠% و١٠% على التوالي بالنسبة للطرق المستخدمة في التحصين بينما أعطى اللقاح الثاني قدرة اقل على الحماية حيث أعطى ٥٠% و٣٠% وصفر% على التوالي عند استخدامهما بالحقن أو خدش الجلد أو بالحمام المائي.

SUMMARY

Traditional methods of control using chemical treatments are not always effective. They can be costly, cause problems of pollution and of drug resistance in the pathogen, and they may be subjected to prohibitive legilisation. Newer techniques of immunizing fish are still in early stages of development. At present it is not possible to assess just how important these will become. They may prove to be more applicable to brood stock

rather than on growing fish, and to intensive systems rather than the more traditional fish farming systems. In the present work, an effort was made to find out an antigen that can protect fry fish against infection with *Ichthiophthirius multifiliis*. Juvenile grass carp fish were immunized against *I. multifiliis*. Two vaccines were developed; heat attenuated vaccine and whole parasite crude antigen. The routes of immunization were; I.P. injection, skin scraping and bath immersion. The percentage of relative protection (PRP) was studied for immunized fish in contrast to non immunized ones. Comparison between PRP afforded by both vaccines were done. A modified method was developed to produce heat attenuated vaccine. The attenuated vaccine gave more protection than the crude one. Fish immunized by the living attenuated vaccine show PRP of 100%, 70% and 10%, for I. P. injection, skin scraping and bath immersion routes of vaccination respectively. While that for fish vaccinated with crude antigen were 50%, 30%, and 00% respectively.

Key words: *Ichthiophthirius Multifiliis Fouquet.*

INTRODUCTION

The ciliate protozoan *Ichthiophthirius multifiliis* is the cause of Ichthiophthiriasis, commonly known as white spot disease which generally occurs in narrow water tanks, in rearing Ponds where there is little or no water change, and in hibernation ponds. The parasite, *Ichthiophthirius multifiliis,* causes sever epizootics in freshwater aquaria, hatcheries and ponds as well as in wild fish populaion (Price & Bone, 1985). The life cycle and activity of trophozoites and their invasive power are temperature-dependent (Richards, 1977). The period from fall to spring is the main out break period in warm water fish because this parasite prefers relatively low temperature (Burkart et al., 1990).

I. multifiliis life cycle which requires no intermediate host, has been described many times, e.g. Van Duijn (1967), can be summarized as; the trophozoites are located beneath the epidermis of the host. The mature ones leaves the host and settles down in the water where it secretes a thin cyst, within which it undergoes rapid division and large numbers of infective tomites are released to invade further new hosts. The optimal duration of full development is 4-7days at room temperature, where the life cycle is temperature-dependant (Hoffman, 1978). After

infection by the parasite, the fish responds by forming a cyst around it. These white spots are easily countable.

Several methods were used to treat the infected fish. From the oldest physical means, Stalk (1956) mentioned that raising the water temperature up to 33°C for 6 hours then cool to 21°C for 3-5 times, this produced 90% relief of the infected cases. Also, other chemicals such as Formaline and Sodium chloride were used to control the disease. (Richards, 1977).

Attention has concentrated around the prospects of preventive immunization as a mean of control depending upon the phenomena of acquired solid immunity for fish surviving infection of *I. Multifiliis* open the field to develop a vaccine against this parasite. (Dickerson and Dawe, 1995).

In the present study living attenuated vaccine and whole parasite crude vaccine were evaluated through three routes of administration "I. P., Skin scraping and bath immersion "Potency of vaccine was tested by challenge infection.

MATERIALS and METHODS

Source and maintenance of *I. Multifiliis*:

Naturally infected grass carp fish acquired from local canals at Kafr El-Sheikh, Egypt, was the source of the initial *I. multifiliis* isolates. The parasite was maintained in finger leng grass carp fish. New parasite isolates from additional infected fish were added to prevent the culture from becoming senescent. Water temperature was maintained at 23-25°C under the same day/night regime. Water quality parameter (NH3, NO3 and pH) were monitored daily by standard test kits (Subasinghe & Sommerville, 1989).

Experimental fish:

Groups of fry grass carp fish, 2.5-3 cm length, 20 fish each, were held in 5L.water tanks at 23-25 °C in a flow-through system with a central biologic filter (Gratzek et. al. 1983), fish were fed once a day. The fish were treated for external parasites with Nitrofurazone and Formalin-Malachite green mixture (Leteux & Meyer 1972) before transfer to the flow-through system. Fish were maintained in the system for 8 weeks before used in the experiments. Water quality parameters were measured daily.

Sampling, fixation and staining of *I. Multifiliis*:

Fresh mounts of the skin, gills and fins were taken and examined according to Santhanam and Srinivasan, (1994), fixed with methyl alcohol in semi-dry state, then stained with Geimsa stain (Conroy and Herman, 1961). Measurements of ten protozoa from each specimens were done in order to determine the maximum and minimum sizes. Identification of the protozoon was carried out according to Stalk (1956) and Schaperclans, (1992).

Antigens preparation;

1- Living heat attenuated vaccine:

Trophonts (tissue stage) were collected from infected fish and allowed to develop into free-swimming tomites according to Dickerson et al. (1989). Tomites were collected and poured through cheese-cloth to remove contaminating mucous and then pelted by centrifugation at 1000 g/ 2min. The supernatant fluid was removed and the cells were re-suspended in 100ml. sterile tap water and pelted as before. The tomites were then re-suspended in 1ml. of water and counted using a haemocytometer (Burkart et. al.1990).

Living infective tomites thrown into the heat attenuation process, temperature of the media containing the parasite raised gradually and incubated at 30°C for 6 hr. then cooled to 20°C. This process repeated three times for the same infective tomites. The degree of attenuation was evaluated by microscopic examination of the parasite vitality after each treatment. (By this way exhaustion without killing of the tomites).

2- Whole parasite crude antigen:

To obtain the parasite trophonts, the infected fish were killed by exposure to a lethal concentration (500mg/L) of MS222 (3-aminobenzoic acid ethyl ester, Sigma chemicals USA). Gently lifting and agitating the fishes skin with forceps loosened any fixed trophonts which were then pelted by centrifugation at 1000g/2min. The cells were resuspended in 100ml. sterile tap water and again pelted by centrifugation (Dickerson et al., 1981). After the supernatant fluid was removed the trophonts were suspended in 1ml. sterile tap water. The collected trophonts sonicated for 5 min. under 150 W. interrupted pulse out-put at 50% duty cycle using a Sonifier cell disrupter (Model W 350, Branson Power Supply Danbury, CT) Protein concentration in the prepared antigen was measured.

Immunization of fish:

Each vaccination trial was repeated three times. Eight groups, 20 each (G1, G2, G3, G4, G5, G6, G7, and G8) of fry grass carp. Each

group of fish were kept in 5L. water tanks at 23 - 25°C during experiment time. (Ling et. al. 1992)

Fish of G1, G2, and G3, were vaccinated with living attenuated vaccine through I. P. injection, skin scraping and bath immersion routes respectively. The used dose was 250 -300 parasite per fish. (after Ling et. al., 1992)

Fish of G4, G5, and G6, were vaccinated with Whole parasite crude antigen through the same previously mentioned routes respectively. The used dose was 250mg/g b.wt. according to Houghton et. al. (1992) The routes of vaccines administration were determined according to Houghton et. al. (1992) for I. P. injection, Cross & Malthew (1993 a) for skin scraping, and Burkart et. al. (1990) for bath immersion route.

Fish of G7 remained as challenged control group.

Fish of G8 kept as negative control group (non vaccinated, non challenged)

Challenge procedure:

Three weeks after vaccination, the vaccinated fish were challenged according to the modified procedure described by Burkart et. al. (1990). Isolated trophont developed in separate wells for 24 hr. at room temp. The number of infective tomites were estimated per well before fish exposure. Ten tested fish were exposed to infective tomites pooled from 15 wells of the incubated plate in 1 L. of water for 30 min. at 23°C.

After challenge, fish were placed into aquaria supplied with individual biological filter. Amonia, Nitrite and pH were monitored daily. Temperature adjusted at 23-25°C under the same day/night regime.

Fish were observed for 10 days after challenge (Ling et. al. 1992)

Determination of vaccine potancy:

The percentage of relative protection (PRP) afforded by the different vaccines, was calculated according to Amend (1981)

$$PRP = 1 - \frac{\%\text{ of mortality of vaccinated fish}}{\%\text{ of mortality of control fish}} \times 100$$

Determination of protein concentration:

The amount of protein in each antigen used was estimated according to the technique of Bardford (1976).

RESULTS

1- Morphology of the different stages of *I. Multifiliis*:

The different developmental stages of *I. Multifiliis* were described and measured in both fresh living wet samples and fixed stained smears during the upsets of the life cycle.

The trophozoites grew from attenuated tomites as shown in fresh wet samples were rounded to oval in shape, smaller in size, the entire protoplasmic mass showed mild lazy movement, The body surface has few number of weakly developed cilia. The invasive power reduced but still present. The parasite looks like the early parasitic stage of vigorous trophozoites 2-3 days after attachment and can not be differentiated from it in the stained samples. Differentiation depends mainly on the movement of the entire protoplasmic mass and the number of attached cilia and their active motion which can be recognized in the fresh living samples.The size remained smaller and the diameter not encreased above 1mm. Diameter of the trophozoite measured 0.6-1 mm., macro-nucleus (0.03-0.1 X 0.2-0.4 mm.) and micro-nucleus (0.1-0.15 mm.). (Fig.1A)

The trophozoites (tissue stage) collected in fresh wet smears from infected fish were round to oval in shape, the entire protoplasmic mass showed in continuous active rotatory movement. The entire surface area of the parasite is ciliated. It measured 1.3-1.6 mm. in diameter. It contain large horse shoe-shaped macro-nucleus (0.05-0.1 X 0.3-0.4 mm.) of vegetative type and small dark stained micro-nucleus (0.15-0.2 mm.) present on the concave side of macro-nucleus. (Fig.1B).

The tomites are pyriform in shape, while they still in the development cyst, (Fig1G) and become rounded in shape when they released from the cyst. They reached 35-50μm in length, the macronucleus is bean shaped not more than .01mm.and present into the posterior portion of the cell. (Fig 1H). They invade fish and penetrates the epidermis of the host with a brisk rotation of the cilia-free tip situated at the anterior pole.

Under the epidermis the tomites build passages and cavities where they grow together. Two to three days after the attachment the parasite grows to spherical form. The epidermis of the host at the site of attack proliferate and the disintegrated cell elements, blood elements and other food remenantes of the parasites accomulated to give the gross picture of the disease, (Fig.2A). From the anterior pole cilia arranged in meridian form. Five to seven days later, The parasite reached maturity (1.5mm. in

diameter). They left the host, settle down in the aquaria (Fig.1D), and devided into two organisms each of them strate to produce a cyst, (Fig.1E).

The developed cyst appear as a protoplasmic mass with indefined shaped wall containing active devided nucleus to give large number of newlly growing nucleulus, (Fig.1E). The cyst becomes spherical in shape increased in size to reach 1.6mm. in diameter and contain early differentiated spindle shaped organisms each containing eccentric nucleus, appeared as dark granules in stained samples, (Fig.1F & 1G).The cyst phase lasts 3-5days under favourable conditions (23-25C). The cyst wall of fully developed one ruptured and Tomites swimme in water towared the surface of water to attack the swimming fish (Fig1H).

2- The clinical symptoms and gross lesions:

The affected fish swim near the water surface, rubbing on the sides or the bottom of aquarium, and swimming violently holding the fins close to the body. White spots, pin-headed size, that goatherd in heavy infection to give white patches of deferent large sizes. These spots distributed on the skin, gills and fins especially the base of the fins. Later on the patches may take the yellow color due to contamination. More sever attacks result in skin detachment. Large number of the affected fish died form asphyxia. The early affected areas are the gills followed by skin of abdominal sides and the base of pectoral fins then the spots spread on other parts of the body. (Fig. 2).

3- The clinical picture of infection in vaccinated fish:

Vaccinated fish which catch infection, table (2) show the previously mentioned symptoms but with different degrees varied from mild, moderate to sever lesion scours. The number of parasites were counted and divided on the body surface area to calculate the infestation rate. In the G1 there was no affected fish. While 6 fish in G2 were mild affected with a parasitic density less than 2 parasite per cm. surface area. In G4 10 fish died within 10 days observation times after challenge. These shown moderate degree of infestation with parasite density 3-6 parasite per cm. surface area. Fish of G3, G5, G6 and G7 were heavily infested with parasite density more than 6 per cm. The disterpution areas varied according to the infestation density where the most common affected areas were skin followed by gills and then the fins base.

Vaccinated fish can tolerate infection with different degrees according to the immunity status produced by the vaccine. Results displayed in table (1) showing that, injection of living attenuated *I.*

multifiliis antigen produce complete protection (100%) with I.P. route, this reflected in the form of absence of moralities. The potency of the same antigen was decreased with change the route of administration, reached to 70% when used by skin scraping route and 10% when applied by bath immersion method.

The use of Whole parasite crude antigen through the above mentioned three routes of vaccination, showing only 50% protection with I.P. route, 30% by skin scraping and no protection (00%) was appeared between fish immersed in this type of antigen.

On the other hand, the challenged control fish show 100% moralities, While the negative control ones show no mortifies during the experiment observation time.

DISCUSSION

The goal of vaccination is to elicit a population of lymphocytes, which upon subsequent exposure to the parasite they proliferate and produce antibodies or effector cells specific to the pathogen. An important aspects of this specificity is the establishment of a long lasting immune memory.

From our work the infective tomites tolerate gradual raising up of temperature up to 33°C, for 8 hours while the sudden rise of temperature from room temp to 32°C kill about 90% of the trophoziotes. This is agreed with that mentioned by Stalk (1956), who stated this method to control the disease by raising the aquarium temperature to 32°C for 6 hours with their repetition three times to kill about 90% of the active trophozoites.Wanger (1960), mentioned that higher temperature increasingly shorten the duration of growth, division and life of the infective parasites. The parasites are incapable of developing below 2-3°C and 28°C. Reichenbach-Klinke's (1973) mentioned that growth of *I. Multifiliis* is completed within 10-14 days at temperature above 22°C while at lower temperature it taken 21 days.

On the other hand El-Khatib (1998), mentioned a physical mean of controlling the parasite by increase of water temperature to 25°C and 30°C and mentioned that the infected fish were recovered from infection within one week with some fish loss. In my openion, the previouse auther optained these resultes not due to the rise of temperature to the mentioed degrees Where, all authors who deals with life cycle or immunization

trials, multiplied parasite and made the infection trials at 23-25°C from those: Stalk (1956), Beur (1958), Beur (1959), Wanger (1960), Van Duijn (1967), Hoffman, (1978), Rice and Bone (1985), Burkart et. al. (1990), Ling et. al. (1992) and Schaperclans, (1992). But I think that El-Khatib (1998) results may be due to the water change, or due to the removal of severely infested fish, or due to the infestation rate was mild and the fish tolerate infection especially this author mentioned that the fish become free of infection one weak later. Ritchards (1977) mentioned that the parasite showing optimum activity at temperature below 21°C but he did not discuss the parasite life cycle in different temperature degrees or mentioned any thing about the effect of high temperature on the trophozoites.

Morphology of mature *I. Multifiliis* and its life cycle stages were as that mentioned by Schaperclaus (1992).

The intensity of the disease in the previous challenged infections was detrmined by the number of the parasite on the host and the lesions appeared on the skin. The vagross effect of the parasite estimated by the number of fish died within 10 days post challenge. Schaperclaus (1992), mentioned that the intensity of the disease is essentially determined by the intensity of attack on the fish and the conditions for the reproduction of the parasites. Bauer (1959), stated that in the case of carp fry, a parasite attack can occur even in the first days after hatching. In about 6-10 days the attack can be so sever that the mortality of early fry sets in. He added that the number of infective young *I. Multifiliis* lethal to carp fry of 6-8 mm. In length, has shown that at 20C, even 50-100 tomites can kill the fry within 1-3 days.

The clinical signs of the affected fish were appearance of the gross lesion "White spots" on the skin and gills, and the violently swimming with closed fins. The degree of infection decreased by increasing aquired immunity of fish. Schaperclaus (1992), mentioed the fully grown parasites can be seen as white grite-like spots on the skin, gills, fins and cornea. The fish react to infestation by rubbing on the bottom, by swimming violently and by holding the fins close to the body. Sever attacks causes detachment of the skin.

In the present study, two antigen preparation (Living attenuated and whole parasite crude antigen) and three routes of administration (I.P., skin scraping and immersion) were used in an attempt to vaccinate grass carp fish against *I. multifiliis.* It was found that I.P. injection of living attenuated tomites provided the most effective means of

vaccination than the other two routs. This came in agreement with the results of Areerat (1974) and Burkart et. al. (1990), who obtained the best protection rates when they used I. P. injection of cat fish with living tomites.

Vaccination using the whole parasite crude antigen showing low degree of protection than the first antigen. The efficacy of this vaccine also was decreased with changing in the route of administration form I. P. to skin scraping route. On the other hand no protection was appeared in fish immersed in this type of antigen this was agreed with the previous findings of Goven et. al. (1980) who used killed tomite by I. P. and immersion routes.

It is worthy to mentioned that skin scraping as a route of vaccination gave protection level in the med-way between I. P. and immersion routes.

Failure of crude antigen to give the promised results with any of the above routes may be due to, the immune response of fish against *I. multifiliis*, depends mainly on the presence of immunogens as a molecule in the living parasites, able to stimulate the immune response of the vaccinated fish. The same phenomena was previously mentioned by Burkart et al. (1990). These immunogens may be also able to induce their stimulating effect when vaccine applied by skin scraping route where the skin surface is already opened. This route stimulate the immune response locally and systemically to induce skin protection barrier but with some limitation.

In immersion technique, fish exposed to the antigen with intact skin that forming the defense against external invasion. This enable the vaccine to stimulate the body defense mechanism as vaccine can not penetrate to the body lymphocytes except the contact through gills and this takes place for a short duration. This is may be the cause of the lowest protection induced by this way of vaccination (the immersion route).

The invasive young of the parasite were not able to penetrate the mucous membrane of the resistant fish.

Bauer (1958), assumed the development of a natural immunity after repeated sub-lethal attacks of common carp with *I. Multifiliis.* The author recorded that the possibility of immunization exists in the case of parasites living in the tissues of its host and not as pure ectoparasites. Hines & spirac (1974), have undertaken specific immunophysiological investigations which confirm the occurance of immunization in his

experimentally infected mirror carp with suplethal dose of *I. Multifiliis.* The author found an increase in the serum immobilization titer between 10-22 day after infestation. The effector mechanisms responsible for immunity to *I. multifiliis* are unknown (burkart et.al.,1990). There was an assumption of specific antibodies and/or lymphocytes are involved. The author cleared that there are large numbers of tissue lymphocytes, leukocytes and eosenophiles distributed under the skin and in the muscels of the fish, these cells can be produce specific antibodies when stimulated through injection or via active coetaneous contact, as the parasite invade and burrowing the skin.This assumption agreed with Cross and Matthews (1993) who stated that in carp fish greeter localized phagocytosis was recorded in immunized fish by neutrophils, macrophages and epidermal filament cells after their challenge with topical application of theronts on the caudal fin .

Pyle & Dawe (1985) also, stated that serum antibodies reach their highest concentration 2I days after a single intra-muscular injection of cilia from *Tetrahymena pyriformis.*

Clark et.al. (1988) and Deckerson et al. (1998) mentioned that sera from *I. multifiliis* immune fish, immobilize the parasite in vitro, with an apparent correlation between specific antibodies and immobilization.

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Table (1)

Evaluation of vaccination trials

type of Vaccine	Groups (each of 20 fish)	Rout of administration	Mortality within10 dpc No.	%	PRP
Living attenuated vaccine	Group 1	I. P.	00	00 %	100 %
	Group 2	Skin scraping	06	30 %	70 %
	Group 3	Bath immersion	18	90 %	10 %
Whole parasite crude vaccine	Group 4	I. P.	10	50 %	50 %
	Group 5	Skin scraping	14	70 %	30 %
	Group 6	Bath immersion	20	100%	00 %
Control Groups	Group 7	non vaccinated and challenged	20	100%	-----
	Group 8	non vaccinated, non challenged	00	00%	-----

dpc = days post challenge . PRP = percentage of relative protection.

Table (2);

The clinical picture of infested vaccinated fish after challenge infection.

Group	Fish No.	Lesion scour	Parasite density Parasites (No./cm. body area)	Distribution of infection on the body		
				Gills	Body	Base of fins
Group 1	00	non	-------	-------	------	-------
Group 2	6	mild	less than 2	+	+	----
Group 3	18	sever	more than 6	++	+++	++
Group 4	10	moderate	3-6	+	++	+
Group 5	14	sever	more than 6	++	+++	++
Group 6	20	sever	more than 6	+++	+++	+++
Group 7	20	sever	more than 6	+++	+++	+++
Group 8	00	non	---------	------	------	-----

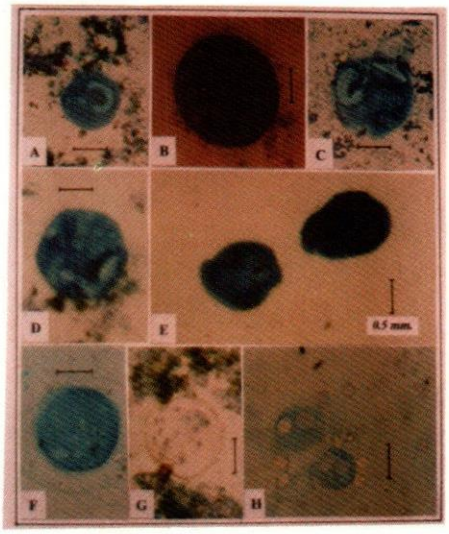

Fig. (1)

A- Growing *I. multifiliis* (X4)
B- Full mature *I. multifiliis* (X4)
C- Falling *I. multifiliis* (X4)
D- *I. multifiliis* start in multiplication (X4)
E- Early developed *I. multifiliis* cyst (X4)
F & G- Developed cyst containing tomites (X4)
H- Released tomites from ruptured cyst (X4)

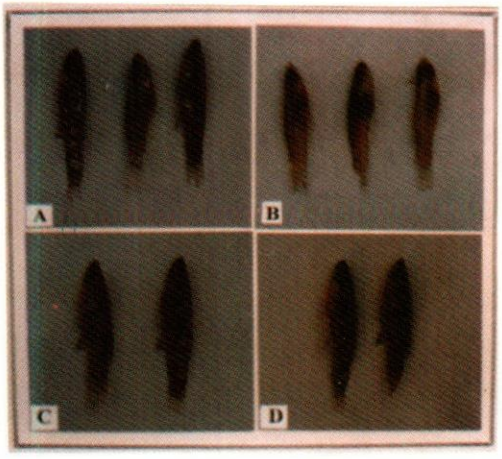

Fig (2),

A- Fish immunized with crude antigen
B- Fish immunized with heat attenuated vaccine
C& D- control groups.