PRELIMINARY STUDY ON ANTIBODY DEVELOPMENT IN *TILAPIA NILOTICA* PETERS AGAINST MICROSPORIDIA

Buenaflor D. Jimenez

Microsporidia are protozoan parasites found to infect tissues of aquatic insects and fishes (Wenyon, 1965, van Duijn, 1973). They are intercellular, spore-producing types and, as multiplication takes place, cause hypertrophy of the parasitized cell. They measure about 2-10u and are among the smallest protozoan parasitizing freshwater fishes (Putz, 1969). Their life cycle is complex and the stage most recognized comprises of a large number of spores which forms whitish cyst within the muscles or gills (Robert and Shepherd, 1974). They are also found to attack other organs such as the reproductive, liver, kidney, spleen and vascular system (Bulla and Cheng, 1976). The infected organs were reported to be opaque and whitish in color. Positive identification is extremely difficult in early infection where spores has not develops yet (Putz, 1969).

In prawn, *Penaeus merguiensis* de Man microsporidia are identified to be the causal organism of white ovary syndrome which causes failure in spawning (Enriquez, et al, 1980). In the golden shiner, greater number of dead eggs in spawning mats of heavily infected fish was observed (Bulla and Cheng, 1976).

The transfer of infection from fish to fish is brought about by the spores which have dropped from the damaged muscle and have been picked up by another fish. The daughter spores are released when the mother structure bursts causing constant infection (Geislev, 1963, Putz, 1969).

The infection contributes to heavy losses of fish and fry and

there is no known treatment except killing the infected fish and thorough disinfection of holding tanks (Geislev, 1963).

Review of Literature

Fish immunology have been reviewed by Sneiszco (1970) and Klontz and Anderson (1970) who gave the fundamentals of the early and recent development.

Immunization work on fishes was started by Duff (1937) through the investigation of the dissociation of furuncolosis bacteria. He observed that fish pathogen lost its virulency and formed characteristically different bacterial colonies when grown in agar containing low concentration of Lithium c chloride. His results led him to investigate further the possibilities of immunizing cutthroat trout against furuncolosis and marked series of immunization attempts.

However, interest on immunization as control of diseases subsided in the 1940's and 1950's. This is because of scarcity of research funds and popularity of sulfa drugs as solution to many problems in hatchery (Anderson, 1974).

Fischkrankheitin (1954 as cited by Anderson, 1974) reported that one to three-year-old carps develop progressively higher agglutinating titer suggesting that older population have greater degree of protection against epizootics. He further suggested that this could be a good method of immunizing carp in commercial basis. Infected fish is released with the young population and those survivors having an acquired immunity are raised as broodstocks.

Laboratory and field immunization against bacterial diseases in fishes have been successful such as *S. gairdneri* against *Aeromonas hydrophila* (Post, 1963, 1966) against *A. salmonicida* (Krantz, 1964, 1970), *Vibrio anguillarum* (Fryer, 1972) and *C. columnaris* (Fujihara and Nakatani, 1971).

Application of immunology in protozoan studies are complicated by the complex antigenic structure of these organisms (Anderson, 1974). Pure cultures or concentrating of protozoan antigen are difficult to obtain without interference of host tissue or protozoan by-product. However, when antigen fractions are

obtained without interference, the techniques for antiserum preparation are identical to that bacteria.

Recently, immunologic techniques against protozoan infections demonstrated that spores of microsporidians are good antigen producing specific antibodies in the rabbit. A comparison of twenty different species of microsporidians produces species-specific antibody that give sensitive reaction with antigen at seven dilution up to 32-128 (Bulla and Cheng, 1976).

Methodology

Experimental Setup

The study utilized twenty 10-liter capacity aquaria. *T. nilotica* measuring 12-15 cm were randomly assigned in each aquarium filled with freshwater and mildly aerated. Fish were tested to ensure that there had been no previous record of microsporidian infection by tube agglutination test of Keleti and Lederer (1974).

Acclimation and Maintenance of Culture

Fish were acclimated to their new environment for one week to ensure that no pecularities of pre-conditioning will interfere with the run of the experiment. Fish were fed with pellets twice daily except on the day before exsanguination at which time no feed was given. Water exchanges was done every four days about two-thirds of its volume.

Experimental Treatments

Four treatments of microsporidian spores (1.5×10^6 spores per ml) were tested for antibody development in *T. nilotica:*

a. control (BPS)
b. one dose of 0.1 ml microsporidian spores
c. 2-doses of 0.1 microsporidian spores
d. 3-doses of 0.1 ml microsporidian spores

Immunization

With the use of a polyethylene disposable syringe, inoculum was injected subcutaneously to avoid injury in fish, taking advantage of the interspace between the thick upper connective tissue of the dorsal ridge leading from the dorsal fin and the softer musculature below. The needle was inserted at a site slightly lateral and anterior to the dorsal fin, was gently thrusts forward and slowly withdrawn to avoid tearing off the tissue and leakage of the inoculum (Fig. 2).

Exsanguination

Exsanguination was done four days after immunization to allow the fish to rest. This was done by cutting off the tail at a location midway between the anal fin and base of caudal fin (Fig 3). The caudal area was thoroughly wiped with an absorbent cotton to avoid mucus and water contaminating the blood. A quick stroke with a sharp blade severed the dorsal aorta and enabled most of the blood to flow into the test tube (Fig. 4).

Coagulation of the Blood

The blood were allowed to stand for 90 min at room temperature to coagulate before centrifugation.

Centrifugation

The blood was centrifuged at 2,000 rpm for 30 min after which the sera were separated from the pellets for agglutination tests.

Tube Agglutination

Titer for specific agglutinating antibodies against microsporidian spores in serum of *T. nilotica* was estimated using tube agglutination test by Keleti and Lederer (1974).

Antisera was diluted 1:10 with phosphate buffered saline, that is 1.0 ml antisera to 9.0 ml of Phosphate Buffered saline (PBS). With a lambda pipette 0.2 ml of diluted serum was pipetted to the first hole of microtiter and to each successive holes

0.1 ml of PBS was delivered (Fig. 5). Antiserum was serially diluted to each of the eight holes and 0.1 ml of microsporidian spores (1.5×10^6 spores per ml) was added in each tube and incubated in $50^{o}C$ for twenty hours.

Results

Tube agglutination test before the start of study confirmed absence of natural antibody against microsporidia (Table 1). Antibody development after subsequent immunization are shown in Table 2. Responses of fish against antigen differed significantly from fish to fish. While some fish are good responders, some are not. For the first batch of fish injected, only one fish showed anti-body development with a titer of 40 and in subsequent treatments all fish developed antibody titers ranging from 140-320 for second treatment and 280-3840 for the third treatment (Table 3).

Antibody development was detected at first sampling, four days after initial introduction of antigen (Fig.1). Initial primary response was rather low in that it required 4-8 days before measurable amounts of antibody was detected in the serum. However, a series of injection later resulted in immediate production of antibody to a higher level than occurred in the primary response.

Antibody development appears to be independent with sex and length. Also, treated fish appeared healthy except one with bulging eye (Table 2) and some darkened exsanguinated blood.

Analysis of variance shows that there is significant difference of antibody development in different treatments doses (Table 3). A dose of 3-0.1 ml showed the greatest antibody titer among treatments.

Discussion

Antibody development among T. nilotica was detected shortly after immunization four days after initial introduction of antigen comparably shorter than that of the trout which when held under ideal condition, would take about 2-3 weeks after initial

stimulation (Anderson, 1974).

In carp and goldfish reared at 28°C, aggulatinating titers showed a rise of agglutinating titer at least four days prior to those kept at 15°C (Cushing, 1942). This high tempe-level could probably explain the reason why immune response of *T. nilotica*, was very fast.

There is some evidence that antiserum was no longer present at the time of second injection as shown in the fast secondary response. This also indicates that the dose used for the first injection was just right to elicit immune response. This is because all of the newly injected antigen is consumed in antigen-antibody complexes and phagocytosed.

Affinity of late antibody is gently increased in the passage of time increasing also the avidity of antibody to antigen. These changes are related to the diverse determinants in the antigen that give rise to a variety of anti-body specificities which appear in the latest period (Bellanti, 1978).

On how antibody is formed in a very short time might be attributed by the fully developed lymphoid system in fish which has both thymus and spleenic components present. It is known that the macrophage of the circulating system are phagocytic and the antigen introduced into the body will be actively phagocytosed. The antigen possibly go through a processing of the macrophage and a message to synthesize antibody is sent to activated lymphocytes and then become the source of specific antibody (Anderson, 1974).

Acknowledgement

I am very grateful to Dr. Gloria L. Enriquez for her encouragement and for making arrangement for my use of the NSRC Laboratory; to Prof. Antonio Mines and IFRD for providing me the *T. nilotica* and pellets and to the NSRC group for sharing me their facilities.

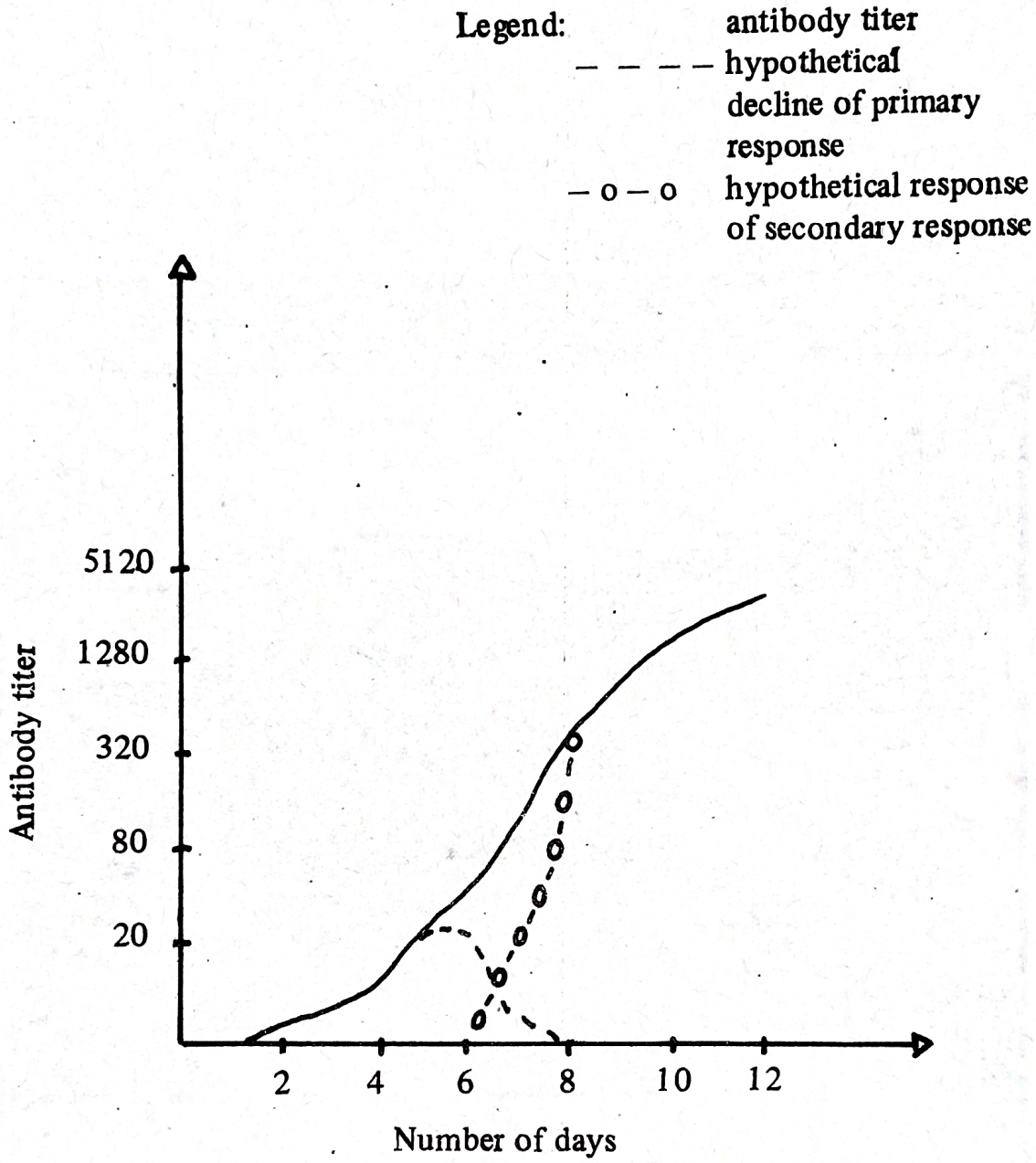

Fig 1 : Antibody titer of T. nilotica after multiple injection of microsporidian spores.

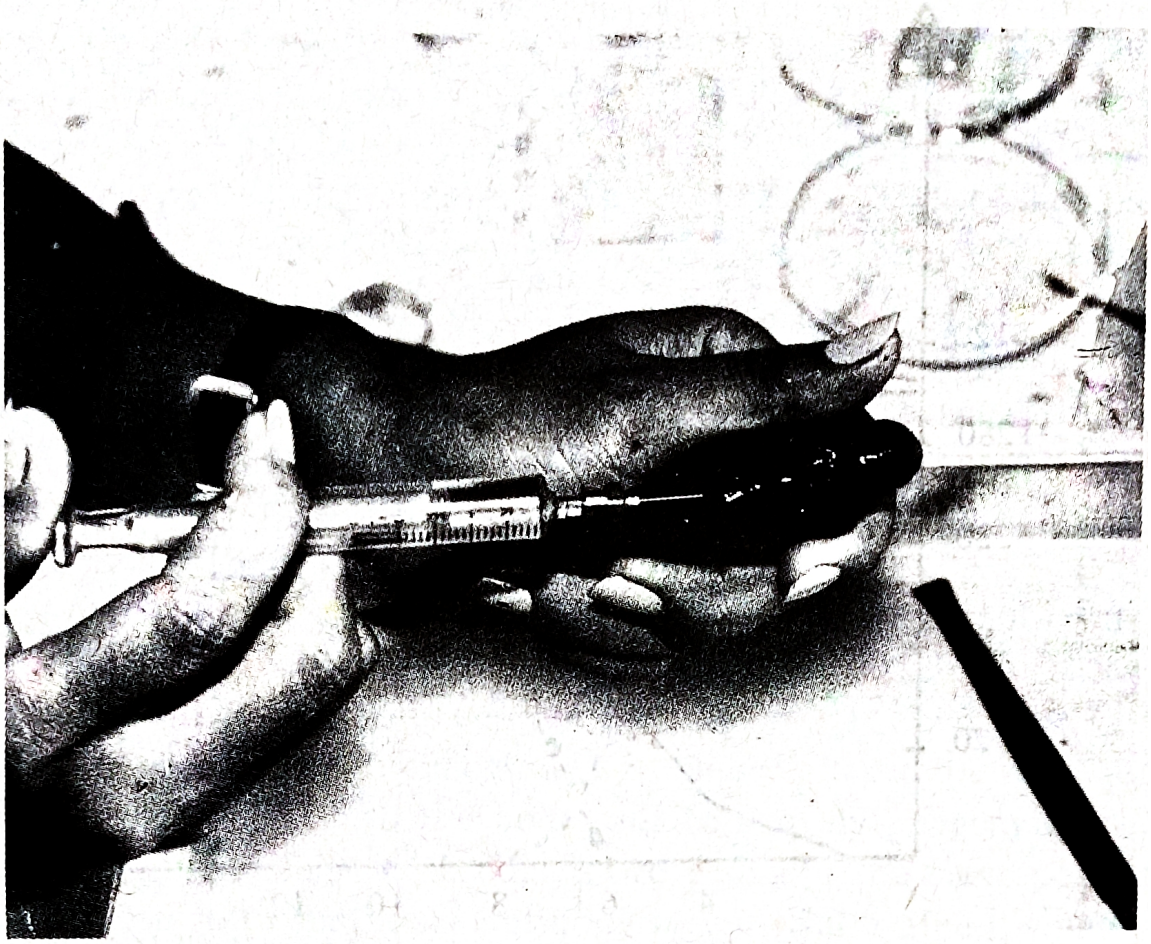

Fig 2. The needle is inserted slightly lateral and anterior to the dorsal fin, gently thrusted forward and slowly withdrawn to avoid tearing off the tissue and leakage of the inoculum.

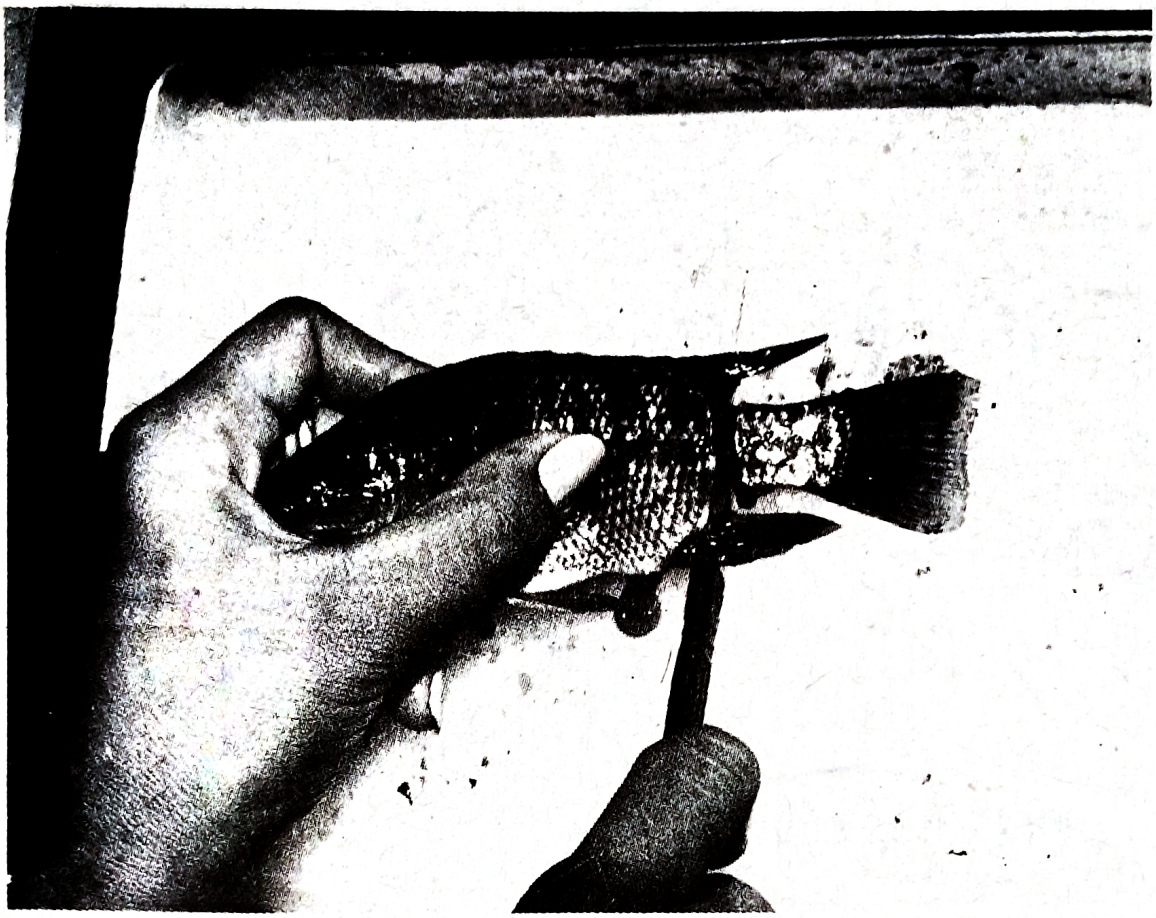

Fig. 3 : A caudal cut at a point midway between the anal fin and base of caudal peduncle to obtain maximum amount of blood.

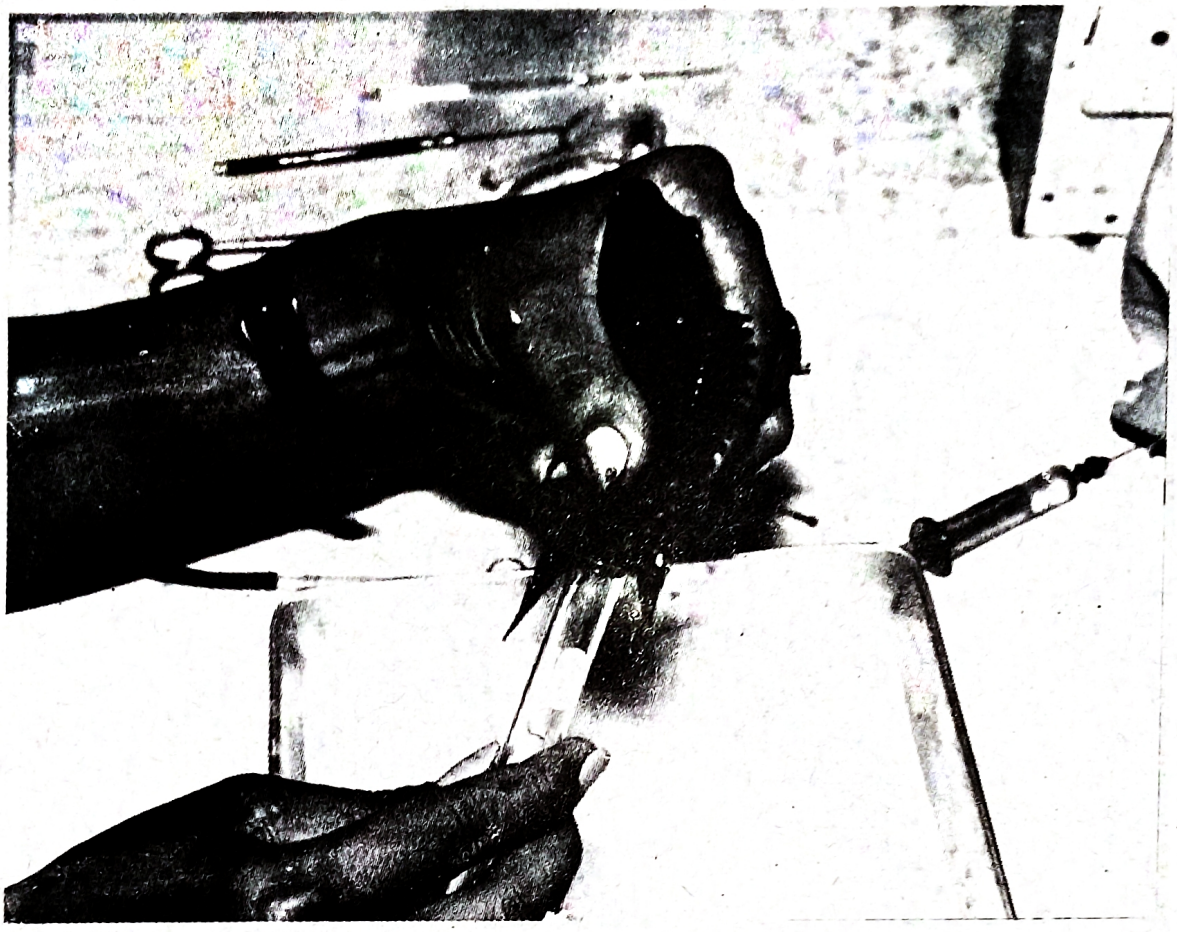

Fig. 4 : Blood is obtained from a caudal cut by tipping the fish and gently squeezing the anterior portion. The tube is gently touched to the dorsal aorta.

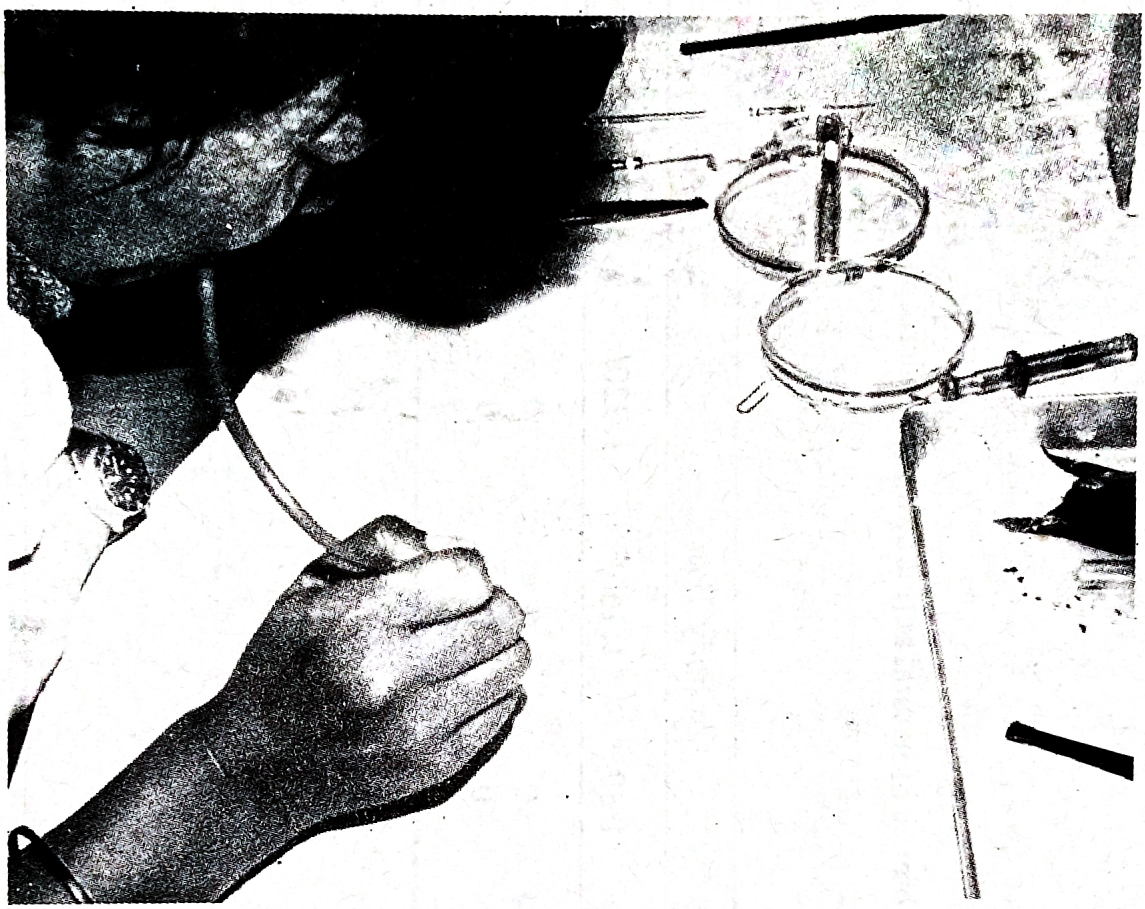

Fig. 5: A microtiter and lambda pipet used for titering antisera.

Table 1. Confirmatory Test of T. nilotica for absence of natural antibody against microsporidia

Sex	Length (cm):	Antibody Titer								
		1:10/	1:20/	1:40/	1:80/	1:160/	1:320/	1:640/	1:1280/	1:2560
o	13.2	0	0	0	0	0	0	0	0	0
0	12.8	0	0	0	0	0	0	0	0	0
0	14.0	0	0	0	0	0	0	0	0	0

Table 2 : Antibody Titer by Tube Agglutination

Exsanguination	Treatments	Antibody Titer	Sex	Length (cm)	Remarks
5-17-82	Control	0	♂	12.0	
	Control	0	♀	14.0	
	Control	0	♂	13.5	
	T_1	0	♀	12.2	
	T_1	40	♂	12.0	
	T_1	0	♂	13.5	
5-21-82	C_2	0	♂	11.5	
	C_2	0	♀	12.5	
	C_2	0	♂	14.0	
	T_2	320	♀	15.0	
	T_2	640	♀	14.2	Blood very dark
	T_2	140	♂	13.5	Blood very dark
5-25-82	C_3	0	♂	12.1	
	C_3	0	♂	13.0	
	C_3	0	♂	14.0	
	T_3	3840	♂	12.0	
	T_3	3840	♂	12.3	
	T_3	280	♂	13.0	Blood very dark, bulging eye

Table 3: Comparison of Antibody Titer Between Treatments

Control	Treatment 1	Treatment 2	Treatment 3
0, 0, 0,	0	320	3840
0, 0, 0,	40	640	3840
0, 0, 0,	0	140	280
0^{a} *	13.3^{a}	366.6^{a}	2653.3^{b}

Table 4: Anova

SV	SS	df	MS	F_c
Treatments	21063844	3	7021281	22.147144**
Error	4438400	14	317028.57	
	25502244	17	7338309.57	
	Ft 3, 14,	.01 = 5.56		

*values with same letters are not significantly different, values with different letters are significantly different.

** highly significant

BIBLIOGRAPHY

Anderson, D.B., 1974. Fish Immunology. Diseases of Fishes, TFH Publishing Inc. 239 pp.

Bellanti, J.A., 1978. Immunology II. W.B. Saunders Co. Philadelphia, pp. 154-159.

Bullah, L. and C. Cheng, 1976. Comparative Pathobiology of the Microsporidia, Plenum Press. N.Y. pp. 154-155.

Cushing, J. 1970. Immunology of Fishes. Physiology of Fishes. Ed. by Hear and Randal,, Vol. 4. The Nervous System Circulation and Respiration. Academic Press. pp. 466-485.

Enriquez, G., C. Beticados and R. Gacutan, 1980. Microsporidian Parasite of the White Prawn, *P. mergueinses* de Man: A Preliminary Report, Nat and Applied Science Bulletin. Vol. 32: 1-4.

Fryer, J.L., D. Nelson and R. Garrison, 1972. Vibriosis in Fish. Progress in Fishery and Food Sciences. Vol. 5: 129-133.

Fujihara, M.P. and R. E. Nakatani, 1971. Antibody Production and Immune Response of Rainbow Trout and Cohe Salmon to Chondrococcus columnaria. J. Fish Res. Board Can 28 1253-2158.

Geislav, R., 1963. Aquarium Fish Diseases. TFH Publishing.

Humphrey, J. and R. White, 1970. Immunology for Student of Medicine.

John, P.W., 1971. Statistical Design and Analysis of Experiments. The Macmillan Co., N.Y. pp. 39-48.

Keleti, G. and W. Lederer, 1974. Handbook of Micromethods for the Biological Sciences. Van Nostrand, Reinhold Co., N.Y.

Klentz and Anderson, 1970. Oral Immunization of Salmonids: A Review on a Symposium on Diseases of Fishes and Shellfish. Am Fish Society Special Publication No. 5, Washington, D.C. 16-20.

Klontz, G.E., J. Reddecliff and C. Heist, 1964. Immune Response of Trout to *Aeromonas salmonicida* Part I. Development of Agglutinating Antibody and Protective Immunity. Prog. Fish Culturist 26: 3-10

Krontz, G. and C. Heist. 1970. Prevalence of Natural Acquired Antibodies Against *A. salmonicida* in Hatchery Trout in Central Pennsylvania. Fish Res. Board Can 27:5.

Lather, A and A. Skillen, 1968. Serological Analysis and Diagnosis, Clinica Chimica Acta Vol. 22:1:31-40.

Post, G. 1966. Response of Rainbow Trout (*Salmo gairdneri*) to Antigen of *Aeromonas hydrophila.* J. Fish Res. Board of Can. 23:1487-1494.

Putz, R. 1969. Parasites of Freshwater Fishes II. Microsporida of Fishes, FDL 20 US Dept. of Interior Washington.

Roberts, R. and C. Shepherd, 1974. Handbook of Trout and Salmon Diseases, Fishing News Books.

Sinderman, C., 1966. Diseases of Marine Fishes. Academic Press.

Sneiszco, S.F., 1970 (ed.) A Symposium on Diseases of Fishes and Shellfish. Am Fish Soc. 526 pp.

Steward, M.W., 1974. Immunochemistry. Chapman and Hall, London. pp. 40-53.

Snedecor, G. 1946. Statistical Methods Applied to Experiments in Agriculture and Biology 4th ed., The Iowa State College Press, Ames, Iowa.

Van Duijn, C. 1973. Diseases of Fishes. London: Ileffe Co., pp. 137-139.

Wenyon, C. 1965, Protozoology: A manual for Medical Men Veterinarians and Zoologists, Hafner Publications Co.