

ON *AEROMONAS HYDROPHILA* INFECTION AMONG CULTURED TILAPIAS : A BIOLOGICAL, HISTOPATHOLOGICAL AND MANAGEMENT STUDY

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ABSTRACT

The prevalence of *Aeromonas hydrophila* infection, the causative agent of motile *Aeromonas* septicemia, was 47.3% among the diseased cultured tilapias. *Oreochromis niloticus* was the more sensitive species to MAS. MAS infected tilapia showed loss of balance, reduced growth, fins and tail rot, ulcer and enlargement of the abdomen. Internally, the organs were congested. The intraperitoneal route was more pathogenic than intramuscular one in the experimentally infected *O. niloticus*. Microscopically different organs showed histopathological changes.

Also, changes in serum biochemical parameters were recorded. The antibacterial activity of Oxytetracyclin (OTC) was evaluated both invitro and in vivo. OTC was the drug of choice for the control and prevention of MAS under laboratory and field conditions. Immunization of tilapia with formalin-killed whole culture vaccine through intraperitoneal route gave successful results.

INTRODUCTION

Tilapia fish are likely to be the most important of all cultured fish in the 21st century. They are farmed in every manner from semiextensive to super-intensive farms (Fitzsimmons, 2000).

Infectious diseases of cultured fish are among the most notable constraints on the expansion of aquaculture and the realization of its full potential (Plumb, 1999; Woo and Bruno, 1999 and Klesius *et al.*, 2000). Bacterial pathogens are the most serious disease problem in tilapia production causing 80% of fish mortalities (Plumb, 1999; Woo and Bruno, 1999; Clark *et al.*, 2000; Shoemaker *et al.*, 2000).

Motile *Aeromonas* Septicemia (MAS) is a more drastic one and distributed world-wide, affecting various species of fish and shellfish, feral as well as farmed fish in both freshwater and sea water, (Inglis *et al.*, 1993; Austin and Austin, 1993; Plumb, 1999; Woo and Bruno, 1999; Fang *et al.*, 2000; Aly *et al.*, 2000 and Azad *et al.*, 2001). MAS is a serious problem for the fish farming industry in Egypt as well as in other countries. The causative agent of MAS, *Aeromonas hydrophila*, is also an opportunistic pathogen of humans causing illness that ranged from mild to dysentery-like diarrhea to meningitis and septicemia particularly when fish is eaten raw or improperly cooked (Inglis *et al.*, 1993). Also, the external clinical signs of the affected fish make them unmarketable.

During the last decade, the interest in protection against fish diseases has grown enormously (Lamers *et al.*, 1985). The lack of effective disease control has the potential of being the chief limiting factor of the realization of highly stable tilapia production (Klesius *et al.*, 2000). The use of vaccines and antibiotics are the two available direct methods to protect farmed fish against diseases. Antibiotics alone will not be enough, but they still have an important role to play in good health management practices (Tafalla *et al.*, 1999 and Clark *et al.*, 2000). Disease prevention by the use of vaccines is the most effective means to reduce disease losses and ensure healthy tilapia production in the new century (Clark *et al.*, 2000 and Klesius *et al.*, 2000).

Therefore, the current investigation was planned to throw light on Motile *Aeromonas* Septicemia as a major disease among cultured tilapias species, as well as the optimal preventive trials to control the disease.

MATERIAL AND METHODS

Naturally Infected Fish: A total number of 150 clinically and grossly diseased tilapia species (50 of each *Oreochromis niloticus*, *Oreochromis aureus* and *Sarotherodon galilaeus*) were collected from different fish farms and transferred to the Central Laboratory for Aquaculture Research, El-Abbassa, Sharkia, Egypt. The diseased fish were subjected to full clinical and postmortem examinations as described by Amlacher (1970) and Lucky (1977).

Bacteriological Examination:

Samples were taken under complete aseptic condition from the affected areas of the external body (fins, tail and gills) and from the

internal organs (liver, spleen, kidney and gonads) and inoculated into tryptic soya broth (Difco) and incubated at 25°C for 24 hours, then further identification by standard microbiological procedures according to Schaperclaus *et al.*, (1992).

Experimental Fish :

O. niloticus was chosen for experimental studies as they are more sensitive species to MAS and the more popular cultured fish.

A total number of 210 apparently healthy *O. niloticus* were collected from El-Abbassa fish ponds and transferred alive to the laboratory to be used in the artificial infection, laboratory trial for Oxytetracyclin and vaccination. Fish were acclimatized to laboratory conditions for 2 weeks, maintained at 25±1 °C in glass aquaria supplied with well-aerated, dechlorinated water. During this time and throughout the experiment, they were fed at 2% of their body weight per day, divided into two diets.

Histopathological Examination:

Samples from affected organs and tissues of naturally infected fish were fixed in 10% phosphate buffered formalin. Parafin sections (5 µ thick) were prepared and stained with hematoxylin and eosin (H&E) and examined microscopically (Roberts, 1989).

Experimental Infection:

A total of 60 apparently healthy *O. niloticus* were divided into 3 equal groups. 0.2 ml dose of 24 hr. broth culture from virulent previously isolated bacterial pathogen of *A. hydrophila* (5×10^5 CFU/ml) were given by intramuscular (i.m.) and intraperitoneal (i.p.) injection for the first and second groups respectively (Schaperclaus *et al.*, 1992). The third group was kept as a control and inoculated with sterile broth (10 fish i.m. & 10 fish i.p.). All the experimentally infected fish were daily noticed for any abnormal clinical signs and mortalities. The dead and clinically diseased fish were subjected to bacterial re-isolation and histopathological examination.

Sensitivity test:

The antibiograms of the recovered pathogen were done using the disc diffusion method. The interpretations of zones of inhibition were estimated according to the limits given by Biomerieux (1984).

Laboratory efficacy trial of Oxytetracyclin (OTC):

A laboratory trial was performed to determine the effect of OTC against infection of *O. niloticus* with *A. hydrophila* (Bowser *et al.*, 1994 and Tafalla *et al.*, 1999). A total number of 30 apparently healthy *O. niloticus* were divided into 3 equal groups. The medicated

diet was prepared by adding powdered OTC at 75 mg/kg fish/day for 10 days (Plumb, 1999). The first group received medicated diet and inoculated i.p. with *A. hydrophila*. The second group received non-medicated ration and challenged with *A. hydrophila*. The third group was left as a control, receiving non-medicated ration and non-challenged. During the 10-d medication period and 15-d post-medication, any adverse clinical signs and mortalities were recorded. All fish were examined for the presence of *A. hydrophila*.

Field evaluation of OTC:

A private farm for intensive production of tilapia suffered from MAS as indicated by our examination was subjected for treatment with OTC (Plumb, 1999). Hygienic disposal of dead fish was done.

Biochemical changes associated with *Aeromonas hydrophila* infection:

Thirty *O. niloticus* were used in this experiment. 10 fish were injected with saline and kept as a control. A total 20 fish were injected i.p. with *A. hydrophila*. Blood samples were collected from clinically diseased tilapia on 3 day post-injection, Biochemical assays were conducted according to Wotton and Freeman (1982).

Vaccine and Vaccination:

Formalin-killed whole culture vaccine was prepared from virulent isolates of *A. hydrophila* used in experimental infection as described by Badran (1987). The sterility and safety tests were performed according to Cardella and Eimers (1990). For parental vaccination, 60 *O. niloticus* weighing from 60- 80 gm were injected i.p. with 0.2 ml of bacterin. A parallel group of control fish (30) was injected with saline. After 2 - 4 - 6 weeks, a challenge infection was done as mentioned by Badran (1987). All the challenged fish (20 of each time) were placed under observation for 21-d, during which the mortality was monitored and the specificity of death was determined by re-isolation of *A. hydrophila* from mortis.

The potency of vaccine was expressed as relative percentage of survival (RPS) using the formula of Cardella and Eimers (1990). The obtained results were statistically analyzed according to Hill (1977).

RESULTS

The prevalence of *A. hydrophila* infection was 47.3% among the diseased tilapia species. The percentages of infection in *O.*

niloticus, *O. aureus* and *S. galilaeus* were 66, 42 and 34 respectively (Table 1).

MAS infected fish showed loss of their appetite, dullness, loss of equilibrium, sluggish swimming at the water surface, detachment of scales, skin erosion and ulcer. The ulcers were usually shallow. Some infected fish had exophthalmia accompanied by hemorrhage or opaqueness of the eye. Fin and tail rot, enlarged abdomen with ascites and if the vent was involved, it might also prolapse. Gills might be congested or pale and anaemic and covered with excessive mucus. The anal and genital regions were also swollen and hyperemic and sometimes small ulcer was noticed near them, other cases showed reddish mouth (Fig. 1).

Internally, the organs were friable and showed a generalized hyperemic appearance (Fig. 2). The liver varied from yellow to dark brown in colour with necrotic foci in some cases and in association with over distended gall bladder with bile. The body cavity contained a bloody and cloudy fluid. The intestine was flaccid, hyperemic, contained yellowish mucus and voided of food. The kidney and spleen were swollen and congested. The gonads were congested, swollen and with abnormal colour in some cases.

A. hydrophila appeared to be Gram-ve, of short motile rods, and **cytochrome oxidase positive (Table 2).**

The distribution of *A. hydrophila* in various tissues and organs of diseased tilapia was demonstrated in Table (3), where a higher percentage was reported from tail and fins (30.17%) followed by gills (20.11%). The lowest was recorded from the ascitic fluid (3.35%).

Experimentally infected *O. niloticus* showed nearly similar clinical signs, with postmortem and histopathological changes to those observed in naturally infected ones. Table (4) showed mortalities pattern among the artificially inoculated fish with previously isolated *A. hydrophila* from naturally infected fish. The i.p. route infection produced a higher mortality than the i.m. route. Re-isolation of *A. hydrophila* was succeeded from all dead and clinically diseased fish. On the contrary, the control group showed neither clinical signs nor mortalities. Furthermore, no *A. hydrophila* was isolated from the control group.

Histopathologically, the infected *O. niloticus* showed desquamation of the upper layer of epidermis with increased number of melanin carrying cells and hyperplasia of mucous cells (Fig. 3). The gills of infected tilapia showed desquamation of epithelial

covering of gills lamellea associated with separation of gill filament (Fig. 4). The liver revealed marked congestion of the central vein (Fig. 5). Also, liver showed infiltration of pancreatic acini by melanomacrophages (Fig. 6). The kidneys showed severe hyaline droplet degeneration (Fig. 7) and coagulative necrosis of renal tubules (Fig. 8).

Regarding to the sensitivity of *A. hydrophila* to different antimicrobials, the bacterial isolates were sensitive to Oxytetracycline, Trimethoprim, Chloramphenicol, Nalidixic acid, Neomycin and Erythromycin. On the other hand, it was noticed that *A. hydrophila* was resistant to Penicillin G, Nitrofurantion and Amoxicillin.

The laboratory trial indicated that the Oxytetracycline incorporated into feed at a dose of 75 mg/kg fish/day for 10 days was quite effective against MAS (Table 5). Survival of fish medicated - challenged or non-medicated non-challenged were higher than that non-medicated challenged fish. No *A. hydrophila* was detected from any fish that survived to the end of the trial in both medicated challenged and non-challenged groups. On the other hand, *A. hydrophila* was isolated from fish that have died during the trial from challenged- non medicated group.

In the field, OTC was found to control MAS successfully when administered at a rate of 75 mg/kg body weight / day for 10 days, where mortalities were declined. Also, the clinical signs were disappeared and the fish returned to normal state of health. Meanwhile, *A. hydrophila* was not isolated from fish after treatment.

With regard to the effect of *A. hydrophila* infectivity on *O. niloticus*, the present results (Table 6) showed a significant differences in hemoglobin (Hb) concentration in-between the control and infected groups. Also, there was a significant decrease in glucose level of the infected group. On the other hand, a liver dysfunction was indicated by a significant increase of both the aspartate transferase (AST) and alanine transferase (ALT) activities. There was non-significant decrease in the total protein.

In the trial of vaccination experiment, table (7), showed the result of immunization of *O. niloticus* fish against *A. hydrophila* using formalin-killed whole culture vaccine through i.p. route. The relative survival percentages were 95, 100 and 100% on challenge at 2, 4 and 6 weeks post-vaccination, respectively. while, the control group had 100% mortality.

DISCUSSION

The causative agent of MAS, *A. hydrophila*, has a worldwide distribution, infecting fishes, birds as well as human. Moreover, MAS is a serious problem for the fish farming industry in Egypt, causing heavy economic losses.

The high prevalence of *A. hydrophila* could be attributed to its presence as a part of intestinal flora of healthy freshwater and marine fish (Newman, 1982; Austin and Austin, 1993 and Plumb, 1999). The present results showed that *A. hydrophila* was the most predominant bacterial species isolated from diseased fish. These results agree with those recorded by Abd El-Rahman (1996) and Sakr (1996) who mentioned that MAS comes first among the diseases infecting fish. Also, there were differences in the susceptibility of tilapias to MAS. Abd El-Rahman (1996) and Abd El-Rahman *et al.*, (2002) cited that *T. zilli* and *S. galilaeus* had higher resistance to bacterial diseases relatively than other tilapias, as *T. zilli* can live at a lower temperature and a higher water salinity than other tilapias.

Regarding to the clinical signs, it was revealed that fish infected with *A. hydrophila* showed loss of equilibrium, fin and tail rot, ulcer and enlargement of abdomen. These results accord with those recorded by Enany *et al.*, (1985); Kabata (1985); Post (1987); Austin and Austin (1993); Inglis *et al.*, (1993); Newman (1993); Stoskopf (1993); Ahmed *et al.*, (1995), Sakr (1996); Abd El-Rahman (1996); Plumb (1999); Woo and Bruno (1999); Shoemaker *et al.*, (2000); Fang *et al.*, (2000) and Azad *et al.*, (2001) who mentioned that the sluggish movement associated with *A. hydrophila* infection was probably the result of frayed and sloughed tail, beside hemorrhagic edematous and ulcerated fins, in addition to anorexia which affected the vital activities of the diseased fish.

The common gross lesions observed in the diseased fish were septicemia in nature as they revealed congestion of all internal organs with abdominal distension and yellowish ascitic fluid. The postmortem lesions were in accordance with the findings of Amlacher (1970); Newman (1982); Enany *et al.*, (1985); Kabata (1985); Post (1987); Roberts (1989); Stoskopf (1993); Inglis *et al.*, (1993); Abd El-Rahman (1996); Sakr (1996); Plumb (1999); Woo and Bruno (1999); Shoemaker *et al.*, (2000) and Azad *et al.*, (2001) that the overdistended gall bladder could be attributed to enteritis and constriction of the common bile duct. Also, the gills were severely

congested and the fins were congested and hemorrhagic. However, many other bacterial infection in tilapia cause the same or similar clinical signs and post-mortem lesions (Plumb, 1999 and Clark *et al.*, 2000) it was therefore considered prudent to make isolates from various tissues of moribund fish.

The morphological and biochemical investigations revealed that *A. hydrophila* was G-ve, with short motile rods and comparable to that recorded by Enany *et al.* (1985), Kabata (1985); Post (1987); Austin and Austin (1993); Inglis *et al.* (1993); Ahmed *et al.*, (1995); Abd El-Rahman (1996); Plumb (1999) and Woo Bruno (1999).

The highest recovery rate of *A. hydrophila* suggested that tail and fins (30.17%) could be the primary entrance for systemic infection as suggested by Enany *et al.* (1985); Sakr (1996) and Azad *et al.* (2001).

The artificial infection, in the present study showed that the i.p. route was more pathogenic than i.m. one. Also, the same clinical signs, postmortem and microscopic findings were similar to that of naturally infected tilapia as reported by Newman (1982); Enany *et al.* (1985); Kabata (1985); Post (1987); Roberts (1989); Stoskoph (1993); Ahmed *et al.* (1995); Sakr (1996); Abd El-Rahman (1996); Plumb (1999) and Azad *et al.* (2001).

Moreover, similar histopathological changes in the infected fish were observed by Amlacher (1970); Kabata (1985); Miyazaki and Kaige (1985); Post (1987); Roberts (1989); Inglis *et al.* (1993), Ahmed *et al.* (1995); Sakr (1996); Plumb (1999); Aly *et al.* (2000) and Azad *et al.* (2001).

It was shown also that OTC was the drug of choice for the treatment of infected *O. niloticus* from MAS, both in-vitro and in-vivo and under both laboratory and field conditions. Similar results were recorded by Kabata (1985); Post (1987); Saleh and El-Naenaey (1990); Badran (1993); Austin and Austin (1993); Eissa *et al.* (1993), Thorpurn and Moccia (1993); Abd El-Rahman (1996); Tafalla *et al.* (1999) and Plumb (1999) who mentioned that OTC has proved a world-wide efficacy to control or treat MAS infection in fish.

However, Shoemaker *et al.* (2000) found that antibiotic treatments had not been effective in eliminating bacterial problems in water re-use systems. They added that a part of the problem with current antibiotic use was that at the time of intervention, the fish were often so sick that they did not eat the medicated feed or that treatments were not of long enough duration. The extensive use of

antibacterials has led to an increase in resistant strains of *A. hydrophila* (Woo and Bruno, 1999).

With respect to the use of hematological parameters and enzyme activities in the early diagnosis of infection, the present results showed marked reduction in Hb, and glucose, while a non significant decrease in total protein was noticed. Liver dysfunction was indicated by significant increase in AST and ALT. Similar findings were reported by Amlacher (1970); Sauer and Haider (1977); Enany *et al.*, (1985); Wakabayashi and Iwado (1985); Mohamed (1987) and Husien and Elias (2000), who reported a destruction of haematopoietic tissues and decrease in blood cell production in infected fish. Also, the progressive loss of serum protein and glucose might be attributed to the increased permeability of renal glomerular capillaries induced by the bacterial toxin.

Nowadays, many commercially available bacterins are used for vaccination against various fish diseases (Newman, 1993). Diseases prevention by the use of vaccine is the most effective means to reduce disease losses and ensure healthy tilapia production (Klesius *et al.*, 2000). The immunization of *O. niloticus* with formalized whole culture vaccine of virulent strains *A. hydrophila* appeared to be more effective and economic for controlling MAS out break among cultured tilapia in Egypt. The non-immunized fish of the control group showed higher mortalities than the immunized ones after challenge. Previous studies showed that i.p. injection of *A. hydrophila* was effective in protecting fish against infection (Newman, 1982; Lamers *et al.*, 1985; Badran, 1987; Stevenson, 1988; Austin and Austin, 1993; Inglis *et al.*, 1993; Ahmed *et al.*, 1995; Plumb, 1999; Woo and Bruno, 1999; Aly *et al.*, 2000; Clark *et al.*, 2000 and Klesius *et al.*, 2000). On the other hand, Lamers *et al.*, (1985) reported that oral application of vaccines was not always effective. Fang *et al.*, (2000) recorded that the bactericidal activity of the specific antisera against virulent and avirulent strains of *A. hydrophila* were different. However, vaccines should not be viewed as a panacea for all ills (Clark *et al.*, 2000). According to Austin and Austin (1993), proper management is essential to success of aquaculture operations, while the inadequate management is the principal factor in triggering disease outbreaks.

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Table (1): The prevalence of MAS in tilapias fish.

Species	No. of examined	MAS	
		No.	%
<i>O. niloticus</i>	50	33	66
<i>O. aureus</i>	50	21	42
<i>S. galilaeus</i>	50	17	34
Total	150	71	47.3

Table (2): Cultural and biochemical reactions of *A. hydrophila* isolated from naturally infected tilapia.

Test	Reaction	Test	Reaction
Gram stain	-ve	Cytochrome oxidase	+
Shape	short rod	Indole production	+
Motility	+	Voges-Proskauer	-
Growth on R-S media	yellow colonies	Nitrate reduction	+
Oxidation-fermentation	F	Methyl red	+
H ₂ S production	-	Gelatin liquefaction	-
Fermentation of Glucose	+	Lactose	+
Manital sucrose	+	Fructose	+
	+	Maltose	

Table (3) : The percentage of *A. hydrophila* from various tissues and organs of naturally infected tilapia.

Fish species	Tail & fins		Gills		Liver		Kidney		Spleen		Ascitic fluid		gonads	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
<i>O. niloticus</i>	19	29.23	12	18.46	11	16.92	11	16.92	4	6.15	3	4.62	5	7.69
<i>O. aureus</i>	17	28.81	13	22.03	10	16.93	9	15.25	5	8.47	2	3.39	3	5.08
<i>S. galilaeus</i>	18	32.73	11	20	11	20	10	18.18	4	7.27	1	0.56	0	0
Total	54	30.17	36	20.11	32	17.88	30	16.76	13	7.26	6	3.35	8	4.47

Table (4) : Mortality rates among *O. niloticus* artificially inoculated with *A. hydrophila*.

Fish Group	Rout of injection	No. of fish	No. of dead	Mortality rate
I	i.m.	20	14	70
II	i.p.	20	18	90
III	i.m.	10	0	0
	i.p.	10	0	0

Table (5) : Laboratory efficacy of Oxytetracyclin for the control of *A. hydrophila* infection.

Fish Group	No. of fish	No. of dead	% of survival
Group 1 (OTC-bacteria challenged)	10	2	80
Group 2 (No OTC-challenged)	10	9	10
Group 3 (No OTC-Non-challenged)	10	0	100

**ON AEROMONAS HYDROPHILA INFECTION AMONG 197
CULTURED TILAPIAS**

Table (6) : Hemoglobin and some serum biochemical parameters in *O. niloticus* infected with *A. hydrophila*.

Item	Control Mean ± S.E.	Infected Mean ± S.E.
Hb. (gm/dl)	7.81 ^a ± 0.28	5.90 ^b ± 0.16
glucose (mg/dl)	51.97 ^a ± 1.54	38.15 ^b ± 2.38
AST (iu/ml)	48.46 ^a ± 1.50	57.51 ^b ± 1.24
ALT (iu/ml)	41.75 ^a ± 0.50	49.40 ^b ± 0.90
Total Protein (gm/dl)	5.19 ^a ± 0.42	4.01 ^a ± 0.40

Means with the same letter are not significantly different.

P<0.05

Table (7) : Showing results of vaccination trial against *A. hydrophila* via intraperitoneal route in *O. niloticus*.

Fish group	Control			vaccinated		
	2 w	4 w	6 w	2 w	4 w	6 w
Time	2 w	4 w	6 w	2 w	4 w	6 w
No. of injected	10	10	10	20	20	20
R.P.S.	-	-	-	95%	100	100

LEGEND OF FIGURES

- Fig. 1: Tilapias infected by *A. hydrophila* showing hemorrhage, scales loss, erosion, corneal opacity and frayed tail.
- Fig. 2: Tilapia infected by *A. hydrophila* showing congested internal organs and abdominal ascites.
- Fig. 3: *O. niloticus* infected with *A. hydrophila*, fin showing desquamation of upper layer of the epidermis which infiltrated with melanin carrying cells and hyperplasia of mucous cells. H & E, x 60.
- Fig. 4: *O. niloticus* infected with *A. hydrophila*, gills showing desquamation of epithelial covering of gills lamellea and separation of gill filament. H & E, x 60.
- Fig. 5: *O. niloticus* infected with *A. hydrophila*, liver showing congested central vein H & E, x 60.
- Fig. 6: *O. niloticus* infected with *A. hydrophila*, liver showing infiltration of pancreatic acini by melano macrophage (MMC). H & E, x 60.
- Fig. 7: *O. niloticus* infected with *A. hydrophila*, kidney showing severe hyaline droplets degeneration. H & E, x 150.
- Fig. 8: *O. niloticus* infected with *A. hydrophila*, kidney showing coagulative necrosis of renal tubules. H & E, x 150.

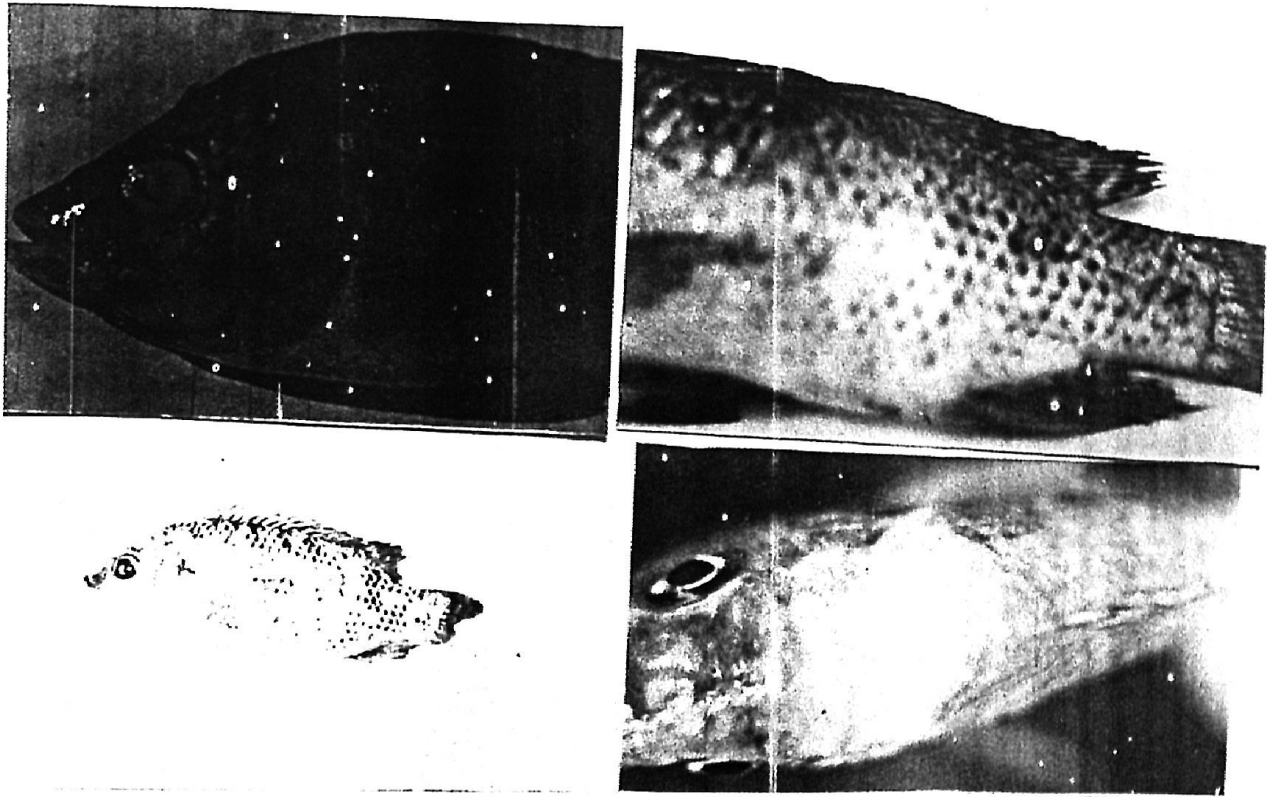


Fig (1)

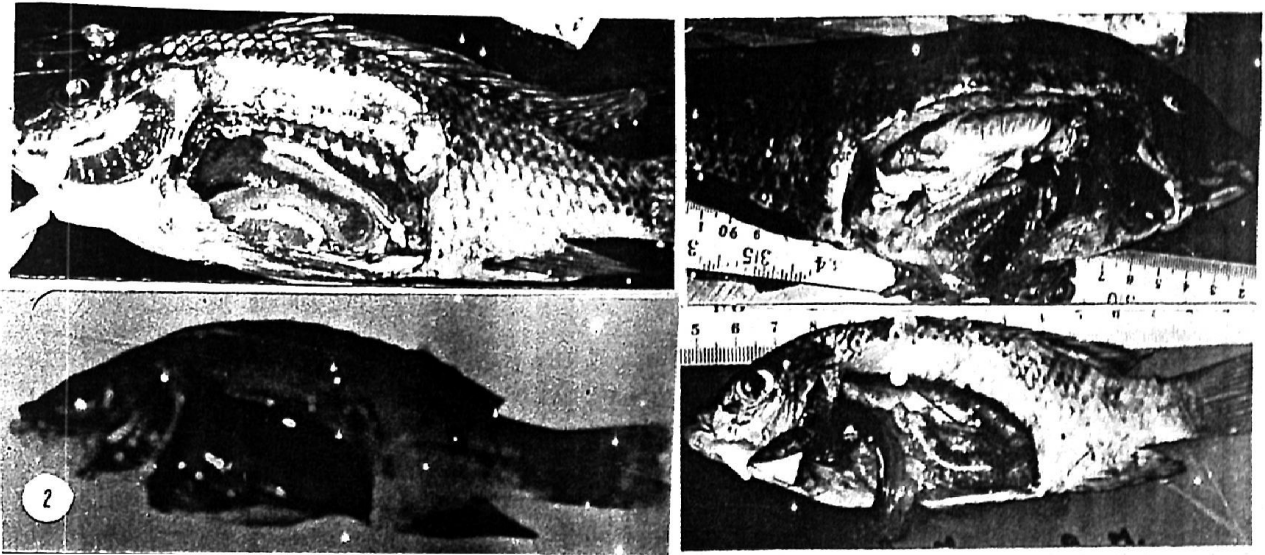


Fig (2)

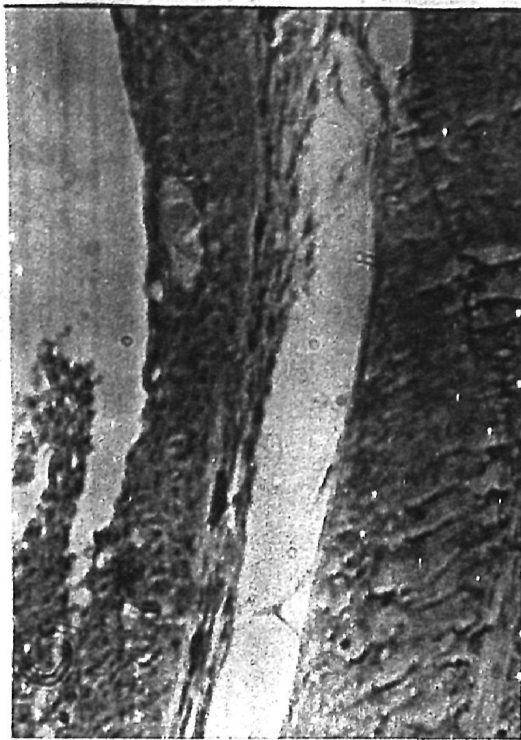


Fig (3)

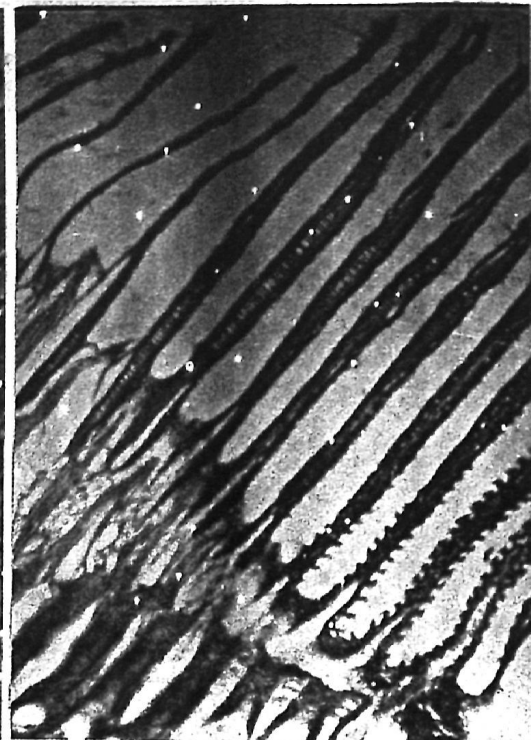


Fig (4)

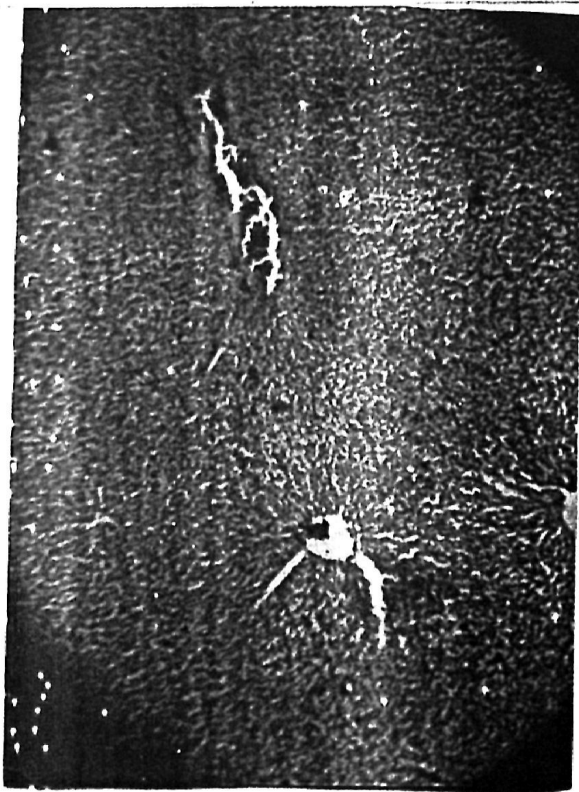


Fig (5)



Fig (6)

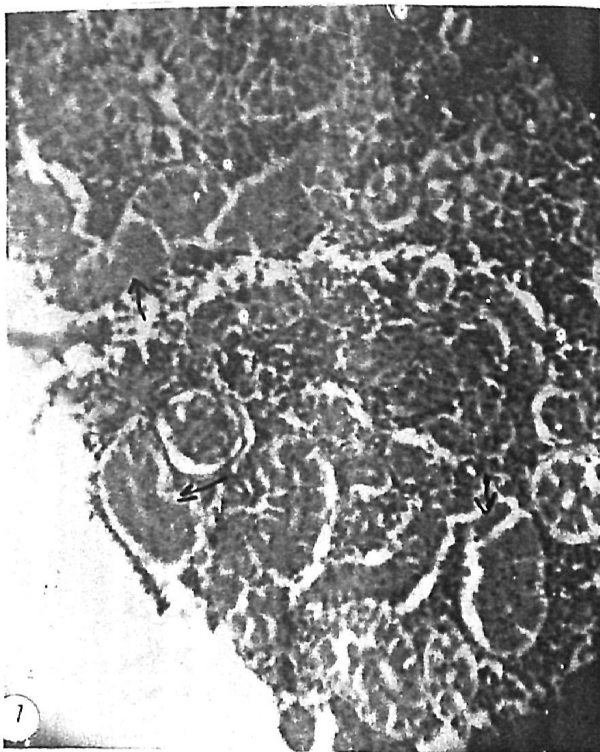


Fig (7)

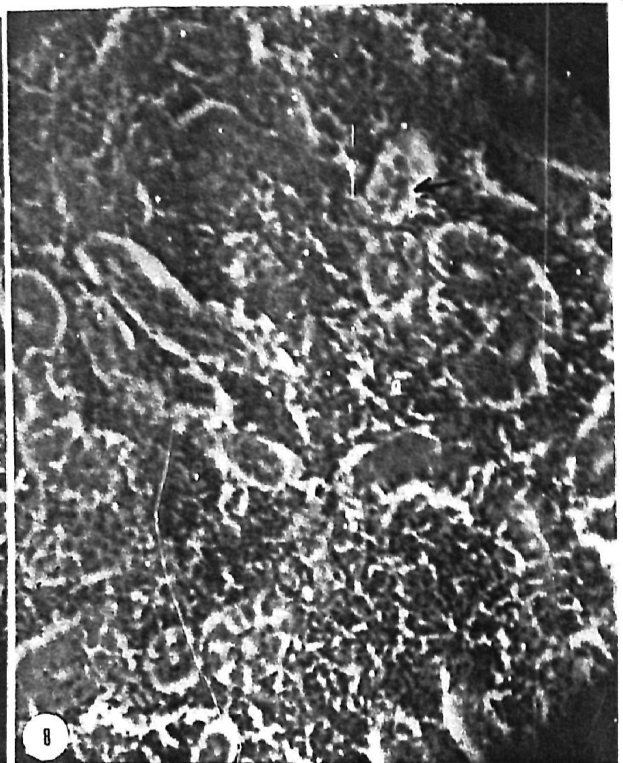


Fig (8)