STUDIES ON ULCERATIVE HEAD SYNDROME AMONG OREOCHROMIS NILOTICUS FISH

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ABSTRACT

Vibrio splendidus, *Aeromonas hydrophila*, *Trichodina centrostigeata* and *T. magna* were isolated from *Oreochromis niloticus* which suffered from ulcer in head region. Various characteristics of *V. splendidus* have been studied. In vitro antibiotic sensitivity, pattern of isolated *V. splendidus* was tested against different chemotherapeutic agents. I/P inoculation of *V. splendidus* broth culture also mixed broth cultures of *V. splendidus* and *A. hydrophila* resulted in 100% mortality, while the challenge by immersion gave no mortalities.

INTRODUCTION

Family *Vibrionaceae* includes two genera, *Vibrio* and *Aeromonas* which are distributed world wide and together constitute the most important species of fish bacterial pathogens (**Inglis *et al.*, 1993**). Vibriosis is one of the major secondary bacterial diseases (**Ruangpan and Kitao, 1991**) and considered as enzootic disease of fish all over the world affecting marine, brackish and occasionally freshwater fishes (**Schaperclaus *et al.*, 1992**) such disease may be represented in the form of focal haemorrhagic ulcer on the mouth or skin surface (**Inglis *et al.*, 1993**). *V. splendidus* has recently been described as new bacteria causing vibriosis in juvenile and adult trubot (**Lupiani *et al.*,1989**). Pathogenicity of *V. splendidus* was confirmed by experimental infection (**Toranzo and Barja, 1990**). However, most if not all the virulence mechanism of these fish pathogens are still unknown (**Woo and Brno, 1999**). Genus *Aeromonas* are capable of causing disease in fish. *Aeromonas hydrophila* have been well documented as fish pathogen, and usually as opportunistic or secondary invaders rather than primary pathogens (**Inglis *et al.*, 1993**) . Ectoparasitic ciliates include different species that are the most common parasites of fishes especially *Trichodina* species. *Trichodinia* Spp. is a frequent problem (**Lom and Dykova, 1992**) and never occurs in large amount on a healthy fish but when the host is weakened by an any stress Trichodiniasis reproduces in massive proportion and start to exert

their pathogenicity (**Lam, 1970;VanAs and Bassan, 1988 and Duran *et al.*, 1991**). Trichodiniasis have been implicated in high moralities of fishes causing severe economic losses in various parts of the world (**VanAs and Bassan, 1987**) and were also responsible for growth inhibition and weight loss in cultured fishes (**Duran *et al.*, 1991**).

The present study was designed for studying the relationship between these fish pathogens for inducing the ulcerative head phenomena and showing the pathogenicity of *V. splendidus* for the first time in *O. niloticus*.

MATERIAL AND METHODS

1. Fish:

(A) Five fish out of 40 live ***O. niloticus*** suffered from ulcer formation in head region were collected randomly from River Nile at Moneab Giza Governorate during August, 2000 with an. average body weight 100 ± 5 g. Fish were examined bacteriologically and parasitologically in Fish Diseases Department, Animal Health Research Institute.

(B) A total number of 72 apparently normal ***O. niloticus*** were collected alive from Nawa farm at Kalubia Governorate, they were kept in glass aquaria supplied with dechlorinated tap water (**Innes, 1966**). Bacteriological mycotic and parasitological examinations were carried out on random 5 fish to ensure that experimental fish were free from the risk of natural infection.

2. Clinical examination:

The naturally infected fish were examined for any abnormalities according to **Plumb and Bowser, (1982)** and **Lom and Dykova, (1992)**.

3. Bacteriological examination:

The natural infected fish were subjected to bacteriological examination according to the methods described by **Inglis *et al.*, (1993)** where aseptically samples from ulcers of such fish were streaked into trypticase soy broth, incubated at 25 C^{O} for 24 hr then loopfulls, from the broth were streaked onto trypticase soy agar (Difco) blood agar as well as some selective media such as Aeromonas base medium (Oxoid) nutrient agar with 2%,4% and 6.5%Na Cl.

4. Biochemical reaction:

According to **Baumann and Schubert, (1965)** and **Krieg and Holt, (1984)**.

5. The in vitro antibiotic sensitivity:

The sensitivity of the isolated *V. splendidus* against antibiotic was carried out in Mueller and Hinton media (Difco) using disc diffusion method

according to **Treagon and Pulliam, (1982)**. The interpretation of the result was undertaken according to **Acar and Goldstein, (1986)**.

6. Parasitological examination:

Smears were taken from ulcers of the naturally infected *O. niloticus* fish they were air dried, then impregnated with 2% aqueous solutions of silver nitrate for 8 minutes followed by rinsing in distilled water, the slide was then placed in white clean dish covered with distilled water and exposed to UV (diffused day light) for about 2 hours, then slides were dried and examined microscopically **Lom and Dykova, (1992)**. Terminology and method of measurement of the components of the adhesive disc followed the uniform specific characteristic system proposed by **Lom, (1958); Wellborn, (1967)** and **Arthur and Lam, (1984)**. Detailed description of the denticles were present in accordance with the method proposed by **VanAs and Bassan, (1992)**. Also body diameter was measured.

7. Experimental infection designs:

Experiment (1): 24 ***O.niloticus*** fish were divided into 4 groups (6 for each), 1st group was injected I/P with 0.3ml of 24 hr old ***V. splendidus*** broth culture contained ($1x10^4$) cell/ml according to **Myhr *et al.*, (1991)** 2nd group was injected S/C with 0.5ml of the same culture while the 3rd group immersed in ($1x10^4$) cell/ml of *V. splendidus* broth culture for 1 hr while the 4th group was kept as a control.

Experiment (2): 24 *O. niloticus* were divided into 4 groups (6 of each) 1st group was injected I/P with 0.3 ml of 24 hr old *A. hydrophila* broth culture contained ($1x10^4$) cell/ml, while 2nd group injected S/C with0.5 ml of the same culture and 3rd group was immersed in broth culture of *A. hydrophila* ($1x10^4$) cell/ml for one hour, 4th group was kept as control.

Experiment (3): 24 *O. niloticus* were divided into 4 equal groups, 1st group was injected I/P with 0.3 ml of mixed broth culture prepared from equal amount (5ml) of *V. splendidus* broth culture and *A.hydrophila* broth culture, each contained ($1x10^4$) cell/ml, while the 2nd group was injected S/C by 0.5 ml of the same mixed culture and 3rd were immersed in the same mixed culture for 1hr, 4th group kept as a control.

Experimentally infected fish were observed for any clinical abnormalities, P/M lesions and mortality rate were recorded during 10 days (the period of experiment).

RESULTS

Result of clinical examination showed that fish suffered from whitish gray superficial ulcer on head region. (Fig.1). The bacteriological examination revealed isolation of 2 types of bacterial isolates, the first one gave identical biochemical reaction to these useful for preliminary identification of *V.*

splendidus (Table 1) It is Gram negative and motile. The colonies were circular, regular medium sized, shiny and translucent The organisms coccoid rods or short bacilli, sensitive to 0/129 (Fig.2), while the second isolate was identified biochemically (Table 2) as *A. hydrophila* which gave green circular colony with dark center on *Aeromonas* base media.

In vitro Antibiogram of the used *V. splendidus* isolate to a variety of antibiotics is to be seen in (Table 3) from this table, it is clear to see that the *V. splendidus* isolate is highly sensitive to oxytetracycline, tetracyclin, garamycin, naldixic acid and nitrofurantoin, sensitive to chloramphenicol and less sensitive to Colistin sulphate, while it resistant to lincomycin, amoxycillin and ampicillin.

The parasitological examination revealed isolation of two species of Trichodinids, *Trichodina centrestigeata*, **Bassan *et al.*, (1983)** and *Trichdinia magna*, **VanAS and Bassan, (1989)**.

T. centrestigeata **Bassan *et al.*, (1983)** is a medium sized with a very high body, surrounded by a finely striated border membrane. The center of the adhesive disc has a characteristic center ridges reaching from 12 to 15 in number of denticles ranged from 27-30. Blade is angular, truncate and slanting backward. The junction of the blade with the central part is narrow. In the same species, tips of the blades are tanget to the border of the adhesive disc. Rays (thorns) are straight or sometimes slightly curved posteriorly, thick at the base and dapering gradually to sharp rounded point central part conical shaped (Fig. 3, Table 4). *T. magna* **VanAS and Bassan, (1989)**, is the largest Trichodinid with disc shaped or saucer- like body surrounded by finely striated border membrane. The center of the adhesive disc is dark and limely granulated. Massive denticles and provided with strongly falcated blades and wedge-like rays. The blade is broad with a round apex. The central part of the denticle is broad at the base and tapers to a rounded point in close association with the preceding denticle rays are long and slightly curved anteriorly They taper from thick bases towards sharp points (Fig. 4, Table 4) Some stages were obtained during division of *T. magna* by binary fission (Fig. 5, Table 4).

I/P inoculation of *V. splendidus* in *O. niloticus* revealed 100% mortality (Table 5) with 48 hr post infection where the fish was suffered from loss of scales, haemorrhage in all body surface (Fig. 6) inflamed vent with small amount of yellowish exudate in body cavity.

In case of S/C inoculation, there was erected scales at lateral sides of fish, white discoloration with large amount of yellowish red exudate in body cavity, haemorrhage in all internal organs with inflammed vent (Fig. 7), with severe ulceration at the site of inoculation which surrounded with haemorrhagic area, while there was no mortality in fish group challenge by immersion, fish was suffered from slight haemorrhage at the body surface which disappeared 96 hr after immersion .I/P inoculation with mixed culture of *A. hydrophila* and *V. splendidus* showed clear respiratory manifestations

that fish try to [illegible] atmospheric air, white discoloration in body surface, haemorrhage in head region, at the base of pectoral fins with protruded vent (Fig.8). The mortality rate was 100%.

While in S/C inoculation of mixed culture, there was haemorrhage at the pectoral and pelvic fin, ulcer formation with loss of musculature at the site of inoculation which surrounded by haemorrhagic area.

During P/M examination there was severe haemorrhage in all organs including gonads moderate amount of yellowish brown exudate in body cavity, the mortality rate was 66.6% (Table 6).

There was no change in fish group which infected by immersion method.

In case of fish group which injected I/P with *A. hydrophila*, the mortality rate was 100% with signs of general septicaemia in liver, spleen, and mesentery), while in S/C inoculation the mortality rate was 50% after 96hr post infection (Table 7) with haemorrhage of internal organs. There were no clinical signs in fish group which challenged by immersion.

DISCUSSION

In most cases where ectoparasitc infestation occurs on fish, there is a possibility that secondary infection by bacteria and fungi may take place (**VanAS and Bassan, 1988**), in the present, study we isolated 2 species of ***Trichodina*** and 2 types of bacteria from naturally infected *O. niloticus* were isolated.

Isolation of *V. splendidus* from ulcer of naturally infected *O. niloticus* for the first time in Egypt agree with **Inglis *et al.*, (1993)** who recorded that more than one genus of Vibrio have been isolated in outbreaks of Vibriosis in fish and shellfish (*V. anguillarium, V. vulnificus, V. splendidus* and *V. pelagius*) also with **Lupiani *et al.*, (1989)** who isolated *V. splendidus* from cultured turbot.

Examination of Gram stained smear from suspected colonies of *V. splendidus* isolates, showed it was Gram –ve, short bacilli (**Myhr *et al.*, 1991and Inglis *et al.*, 1993**).

Biochemical reactions and other growth characters of *V. splendidus* were summarized in (Table 1), the result basically agrees with that reported by (**Lupiani *et al.*, 1989**) except gelatin hydrolysis and VP reaction which agree with **Myhr *et al.*, (1991)** who reported that VP was 4/36 and **Baumann and Schubert, (1965)** who reported that *V. splendidus* hydrolysed gelatin.

The biochemical reactions of *A. hydrophila* were shown in (Table 2) agree with **Krieg and Holt, (1984)**.

Sensitivity of *V. splendidus* to some chemotherapeutic agents were shown in (Table 3) and this may agree with **Myhr *et al.*, (1991)** who reported that *V. splendidus* is senstive to oxytetracycline and resistant to oxolinic acid

while of **Inglis *et al.*, (1993)**, reported that *V. splendidus* is resistant to ampicillin, oxytetracycline and streptomycine.

In our present work *T. centrostigeata* was isolated from naturally infected ***O. niloticus*** as reported by **Nativided *et al.*, (1986)** who isolated *T. centrostigeata* from *O. niloticus* in philippines, *T. centrostigeata* was originally described from cichlid in South Africa (**Bassan *et al.*, 1983**) it has been recorded from Eastern caprivi (**VanAs and Bassan, 1992**), wild and cultured freshwater fishes in Taiwan (**Bassan andVanA,s 1994**). The body dimensions of the present specimen fall within the range of those previously reported from Taiwan as well as the type population from South Africa. From the previous data it can be confirmed suggestions that *T.centostigeata* is a parasite endimic to Africa which at some stage was translocated to Taiwan via the fish introduction from the African contiment.

T. magna seems to be widely distributed in Africa. This parasite was first recorded in South Africa by **VanAs and Bassan, (1989),** then by the same author in the Zambezi River system (1992) and **Ali, (1992)** our specimens recovered from ***O. niloticus*** nearly agree well with the descriptions of **VanAs and Bassan, (1989) VanAs and Bassan, (1992) and Ali, (1992).**

During the division by binary fission, the first sign is a thickening of radial pins of the mother individuals (Fig. 5) **Lom and Dykova, (1992).** The occurrence of stressors such as parasitism enabled Vibrio to produce infection in form of haemorrhagic ulcers on the mouth or skin surface (**Inglis *et al.*, 1993**).

For the best of our knowledge's the pathogenicity of *V. splendidus* in *O. niloticus* has not been studied yet the experimental infection of *O. niloticus* with *V. splendidus* by S/C route gave 66.6 % mortality with signs of anorexia, beginning of ulcer formation in head region, severe ulceration at the site of inoculation Fig. (6), these this may nearly agree with **Inglis *et al.*, (1993)** who reported that *V. anguillarum* infection may be subdermal also **Abd El Gaber *et al.*, (1997)** who studied the pathogenicity of some *Vibrio Spp in O. niloticus as V. anguillarum,V. ordalli,V. damselea and V.vulnificus.*

The P/M examination showed that intestine was swollen filled with mucoid liquid, large amount of reddish liquid in the peritoneal cavity with inflammed vent (**Luipiane *et al.*, 1989**).

Also mortality rate was66.6% in fish group which injected S/C by mixed culture of *A. hydrophila* and *V. splendidus*).

In case of intraperitoneal inoculation of *V. splendidus* or mixed culture of *A. hydrophila* and *V. splendidus* the mortality rate was 100% within 48hr post infection (Fig.8) there were no changes in the internal organs with absence of reddish exudate.

The virulence mechanism of *V. splendidus* is still unknown (**Woo and Bruno, 1999**) In case of fish group which infected by immersion in *V. splendidus* broth culture, the mortality rate was zero, no gross lesion except

slight haemorrhage on body surface which disappeared after 96hr post immersion.

This result may indicate that *V. splendidus* need predisposing factor as reported by **Inglis *et al.*, (1993)** who reported that the occurrence of stresses such as ectoparasitism enabled Vibrio to produce infection in the form of haemorrhagic ulcer on the mouth or skin surface.

In such cases the mortality of fish may occur due to secondary infection **(Van As and Bassan, 1988)**.

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Table (1): Biochemical reactions of *V. splendidus*

Test	Result	Test	Result	Test	Result
Gram reaction	- ve	Indole	- ve	Lactose	+ ve
Oxidase	+ ve	MR	+ ve	Xylose	+ ve
Catalase	+ve	VP	- ve	Arabinose	- ve
Motility	+ ve	Phenylalanine	- ve	Mannitol	- ve
H2S on TSI	-ve	Gelatin	+ ve	Growth in NaCl at 2% at 4% at 6.5%	+ve + ve - ve
Citrate	+ ve	Anaerobic growth	- ve		
Urea	- ve	Sugar fermentation			
Hydrolysis of Tween-80	+ ve	Glucose	+ ve	Sensitivity to 0/129	+ ve
Nitrate	+ ve	Sorbitol	+ ve		

Table (2): Biochemical reactions of *A. hydrophila.*

Test	Result	Test	Result	Test	Result
Gram reaction	- ve	Indole	+ ve	Glucose	+ ve
Oxidase	+ ve	Urea	- ve	Sorbitol	- ve
Catalase	+ ve	VP	+ ve	Mannitol	+ ve
Motility	+ ve	Gelatin	+ ve	Sucrose	+ ve
Growth at 37C°	+ ve	H2S production	- ve	Arabinose	+ ve
Simmon's Citrate	+ ve				

Table (3): Invitro antibiotic sensitivity listing for *V. splendidus* isolate

Antibiotic disc	Sensitivity
Oxytetracycline OT (30µg)	+++
Tetracyclin TE (30µg)	+++
Garamycin GM (30µg)	+++
Nitrofurantoin F(300µg)	+++
Chloramephenicoll C(30µg)	++
Naldxic acid NA (30µg)	+++
Colistin Sulphate CT (10µg)	+
Ampicillin AML(10µg)	-
Lincomycin. MY (2µg)	-

Highly sensitive +++
Intermediate sensitive ++
Less sensitive +
Resistance

Table (4): Morphological data (in µm) of *T. centrostigeata* and *T. magna* from ulcer of naturally infected *O. niloticus* fish.

	T. centrostigeata Bassan *et al.*,1983(n=25)	T. magna VanAS and Bassan 1989(n=25)
Diameter of		
Body	45.7 (44-48)	61.2 (50-68)
Adhesive disc	37.3 (36-40)	52.6 (45-59)
Denticular ring	21.2 (17-36)	32.2 (27-36)
Number of Denticles	28(27-30)	24 (24 -29)
Radialpins/denticle	6 - 8	9 (8 -11)
Dimension of a denticle		
Blade	6 (4-8)	7.2 (6-8)
Central part	2 (1-3)	2.4 (2-3)
Ray	4.2 (4-6)	10.5 (9-12)
Length	4.6 (4-6)	9.8 (9-11)
Span	13.7 (12-15)	19.8 (19-21)
Width of the border membrane		
Central of adhesive disc	With central ridges	Dark and finely granulated
Adoral spiral	400^{O}	405^{O}

Table (5): Virulence of *V. splendidus* in *O. niloticus*.

Group	No. of fish	Route of inoculation	Bacterial culture	Dose cell/ml	Days post infection 0 24hr 48hr	Mortality %
1	6	I/P	V.splendidus	0.3ml ($1x10^4$)	6/6	100
2	6	S/C	V.splendidus	0.5ml ($1x10^4$)	4/6	66.6
3	6	Immersion	V.splendidus	$1x10^4$cel l for 1 hr	0/6	0
4	6	I/P	Saline	0.3	0/6	0

Table (6): Mortality rate in *O. niloticus* experimentally infected with mixed culture (*V. splendidus and A. hydrophila*)

Group	No. of fish	Route of inoculation	Type of culture	Dose cell/ml	Days post infection 0 24hr 48hr	Mortality %
1	6	I/P	Mixed culture of A. hydrophila and V. splendidus	0.3ml ($1x10^4$)	6/6	100
2	6	S/C		0.5ml ($1x10^4$)	2/6 4/6	66.6
3	6	Immersion		$1x10^4$cel l for 1 hr	0/6	0
4	6	I/P	Saline	0.3	0/6	0

Table (7): Virulence of *A. hydrophila* in *O. niloticus*.

Group	No of fish	Route of inoculation	Type of culture	Dose cell/ml	Days post infection 0 24- 48- 96hr	Mortality %
1	6	I/P	A. hydrophila	0.3ml ($1x10^4$)	2/6 –6/6	100
2	6	S/C		0.5ml ($1x10^4$)	1/6 2/6 3/6	50
3	6	Immersion		$1x10^4$cel l for 1 hr	0/6	0
4	6	I/P	Saline	0.3	0/6	0

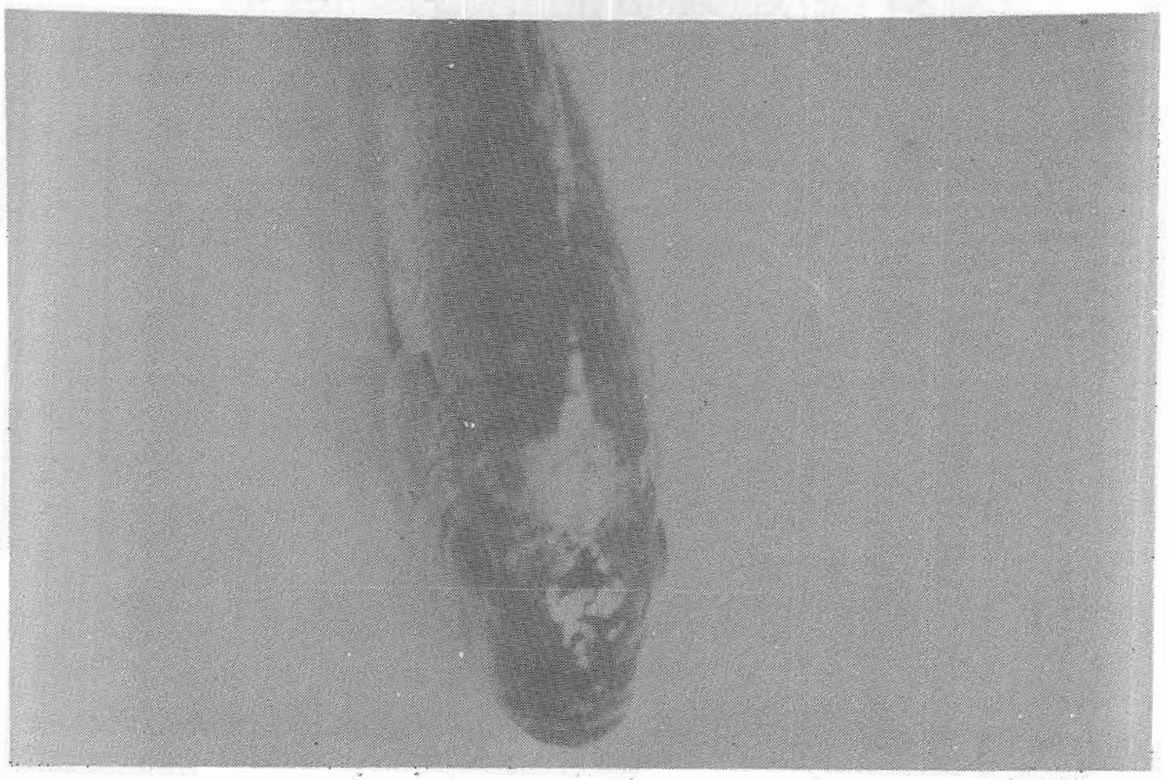

Fig (1): *O. niloticus* suffered from ulcer formation on head region (Naturally infected)

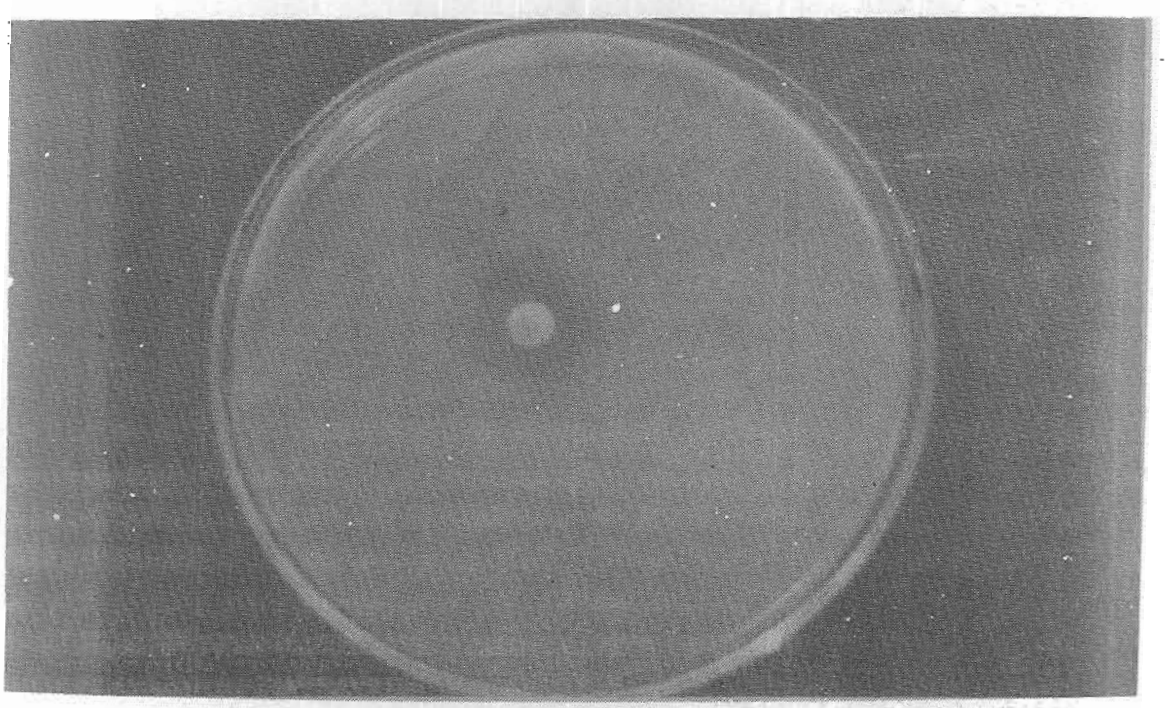

Fig (2): Sensitivity of *V. splendidus* to vibriostate 0/129.

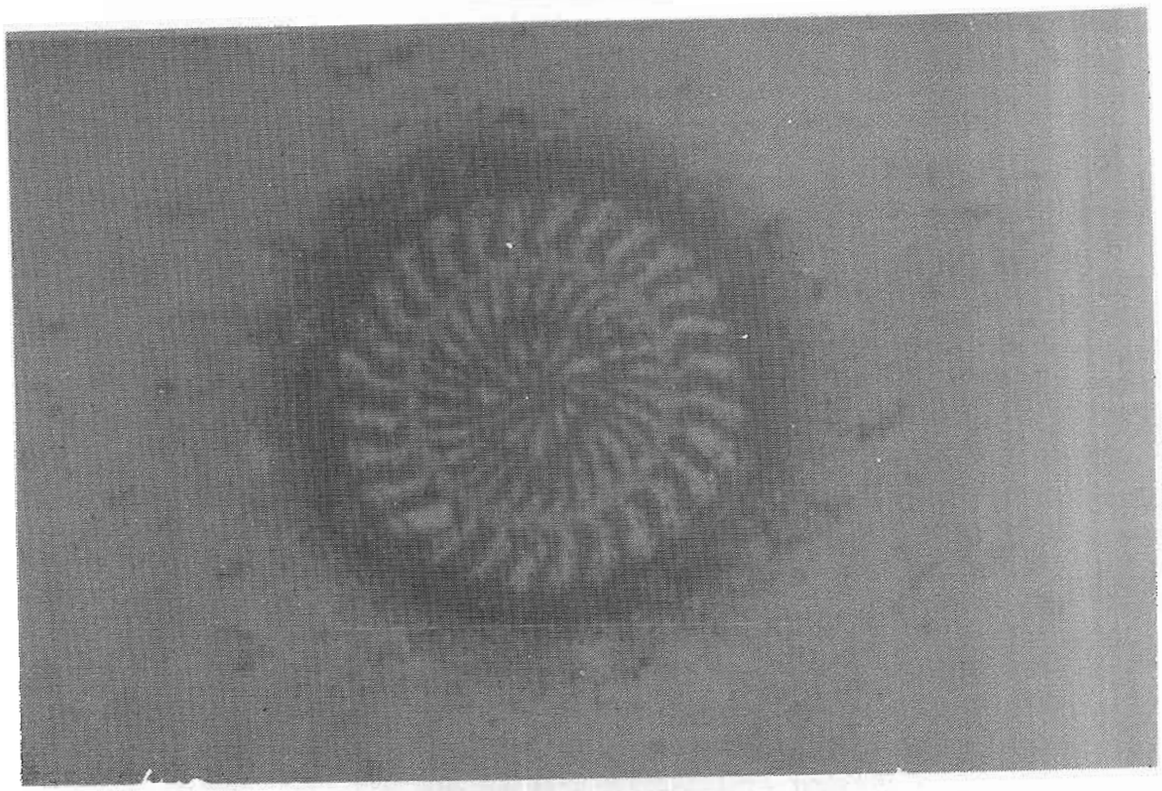

Fig.(3): *T. centrestigeata*

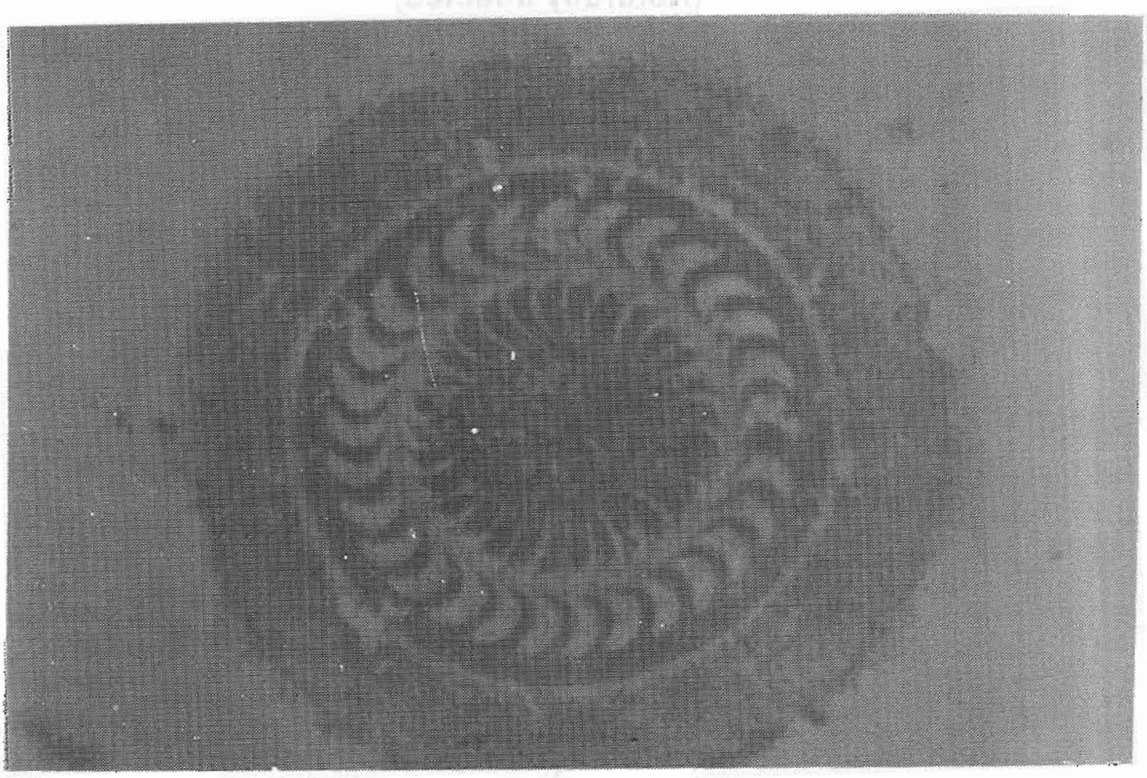

Fig.(4): Mature old specimen of *T. magna*

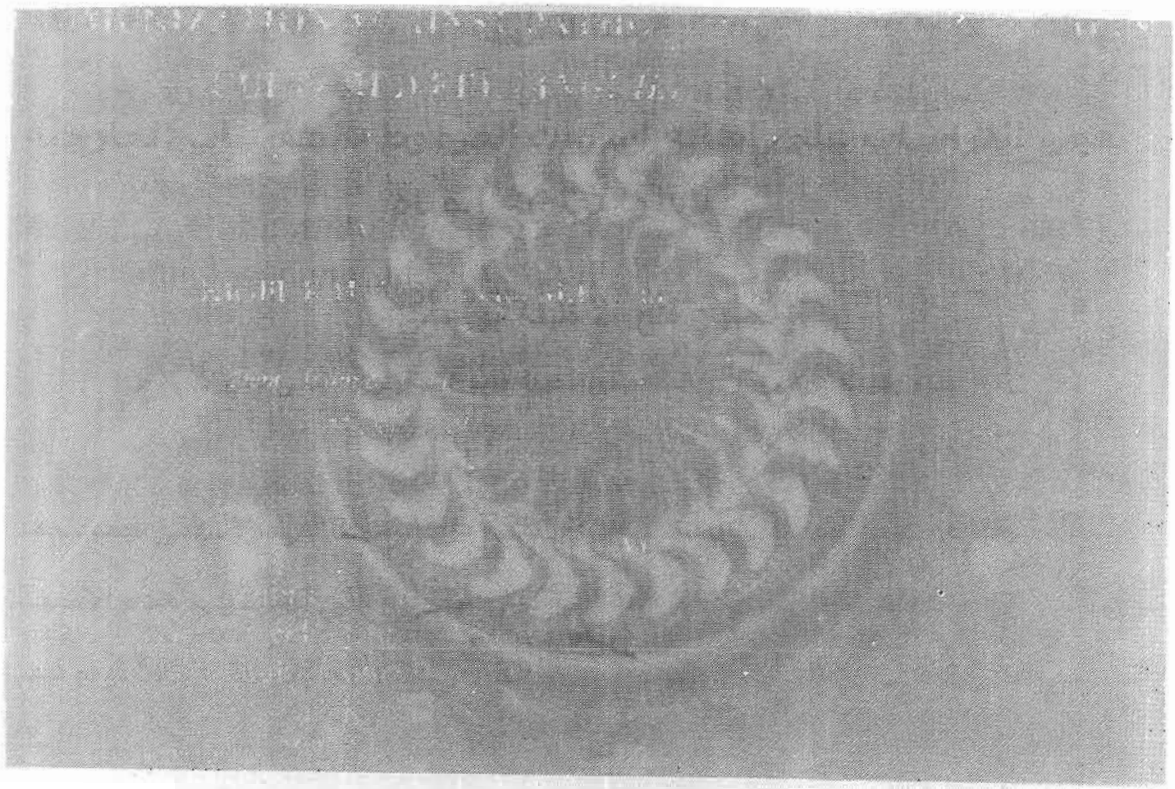

Fig.(5): *T. magna* prior to binary fission as appearing from radial pins arrows

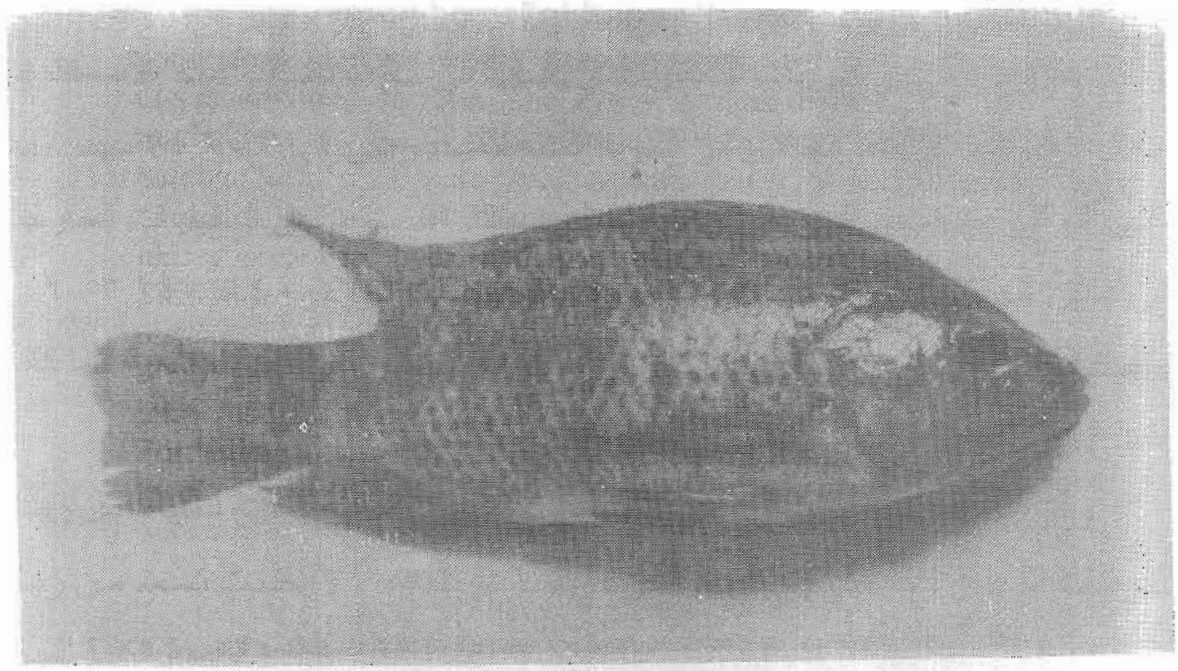

Fig. (6): *O. niloticus* injected I/P with *V. splendidus* after48 hr of infection. While in S/C inoculation of mixed culture, there was haemorrhage at the pectoral and pelvic fin, ulcer formation with loss of musculature at the site of inoculation which surrounded by Haemorrhagic area.

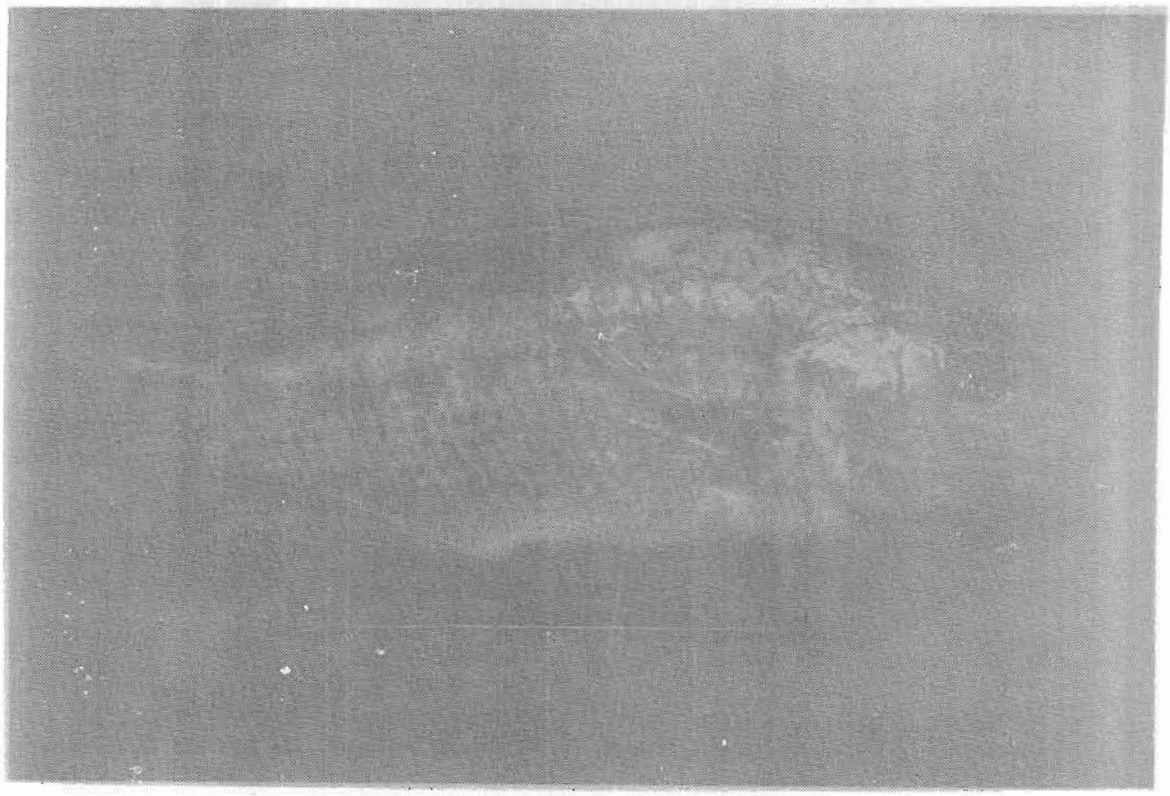

Fig.(7): *O.niloticus* infected S/C with *V. splendidus* showed ulcer formation and inflamed vent

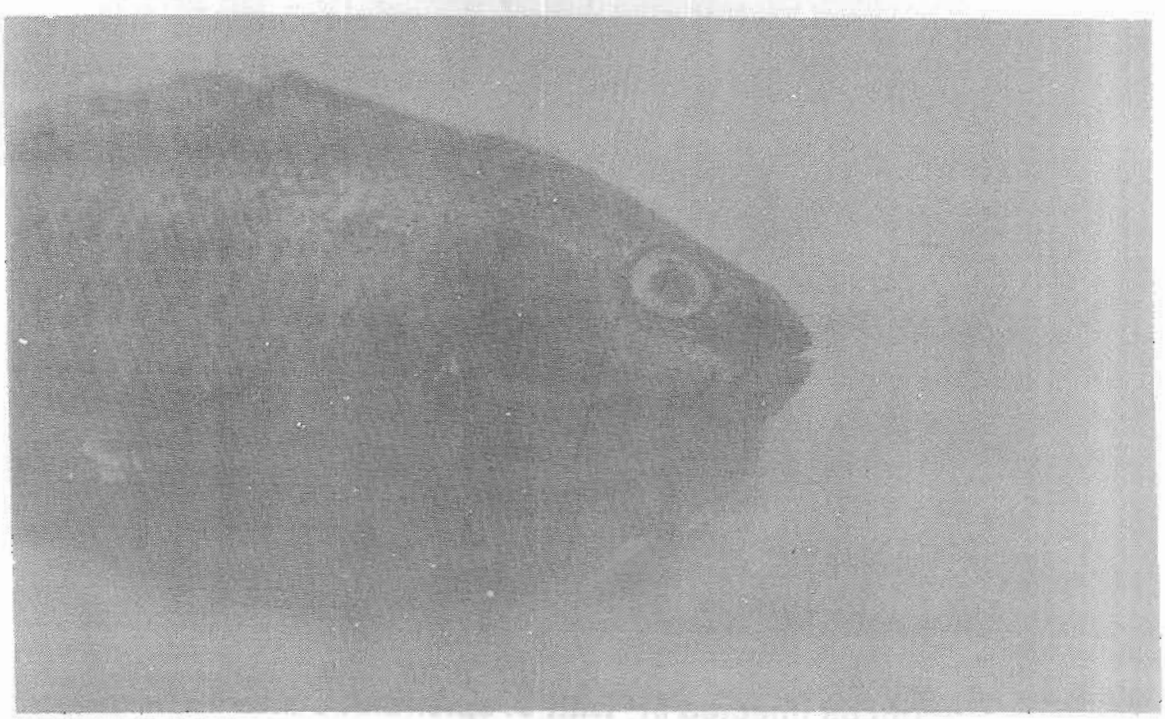

Fig.(8):*O. niloticus* experimentally infected I/P by mixed culture of *A. hydrophila* and *V. splendidus*.

الملخص العربي

الميكروبات المسببة لظاهرة تقرح الرأس فى أسماك البلطى

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تمت هذه الدراسة بقسم بحوث امراض الاسماك بمعهد بحوث صحة الحيوان أثناء تجميع اسماك البلطى النيلى وجد ان عدد خمسة اسماك من أربعون سمكة مصابة بتقرحات فى منطقة الرأس، و أثبت الفحص البكتريولوجى إصابتهم بميكروب (الفيبروسبلنديكس) لاول مرة فى مصر وميكروب (الايروموناس هيدروفيلا). كما اثبت الفحص الطفيلى اصبتها (بالتريكودينا سنتروجانا) و (التركودينا مجنا) و اثبت اختبار الحساسية حساسية (الفيبرواسبلنديكس) العالية الى (الاوكس تتراسيكلين) و (التتراسيكلين) و (الكلورمفينيكول) و مقاومتها الى (النيتوميسين ، الاموكسيلين) وقد أجريت العدوى الصناعية بميكروب(الفيبرواسبلنديكس) بالحقن فى الغشاء البريتونى و ادت الى 100% نفوق اما الحقن تحت الجلد ادى الى 66.6% نفوق.اما العدوى بطريقة الغمس لم تؤدى الى اى نفوق. وايضا العدوى الصناعية بكلا من (الفيبروسبلنديكس) و(الايروموناس هيدروفيلا). أعطى نسبة نفوق 100% عند الحقن فى الغشاء البيروتونى و أعطى 66.6% عند الحقن تحت الجلد. أما العدوى بطريقة الغمس لم تؤدى إلى آي نفوق او ظهور أعراض مرضية.