OCCURRENCE AND PATHOGENICITY OF *SERRATIA MARCESCENS* AT FISH FARMS IN KAFR EL-SHEIKH GOVERNORATE

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ABSTRACT

An investigation was carried out for unusual disease outbreaks occurred on 5 commercial oreochromis niloticus (O.niloticus) fish farms during the peroid 2003 – 2004 , in 3 Districts in Kafr El-Sheikh Governorate. Affected fish exhibited anorexia , sluggishness , variously sized areas of haemorrhage on the skin , corneal opacity and high mortality up to 21.4% . Bacterial isolates from the moribund fish were identified as ***Serratia marcescens (S.marcescens)*** *by biochemical tests revealed 28 isolates. The virulence properties of this isolates , studied revealed that were highly pathogenic for fish with* LD_{50}*s ranging from* $5x10^3$ *to* $1x10^5$*. Moreover , the isolates were pathogenic for mice with* LD_{50} $1.6x10^6$*. Histopathological alterations of the natural and experimental infections are described . The antibiogramme of the isolated* ***S.marcescens*** *was investigated . This study is considered the first record of* ***S.marcescens*** *infection in O.niloticus fish in Egypt .*

INTRODUCTION

Bacteria are important pathogens in both wild and cultured fish and are responsible for serious economic losses . Some may cause primarily a surface (skin or gill) infection; most can cause systemic disease. A wide array of bacteria cause infections in freshwater fish. Most pathogens are Gram-negative rods. The only members of the family Enterobacteriaceae which are currently recognized as substantiated fish pathogens are *Yersinia ruckeri* , *Edwardsiella tarda* and *Edwardsiella ictalurii* . However , other

enterobacteria such as *Proteus,Citrobacter,Hafnia, Klebsiella* and *Serratia species* have occasionally been associated with fish disease outbreaks ***(Bejerano et al., 1979; Llewellyn, 1980; Sato et al., 1982; Gelev et al., 1990; McIntosh and Austin, 1990; Nieto et al., 1990; Gado and Abd El-Aziz, 2003 a,b)***.Therefore,the possible role of these bacteria in aquac-ulture remains to be determined.

In July 1992, Baya et al. succeeded in isolation of a red pigmented bacterium which resembled a *Serratia spp.*in pure culture from fish in Back River. Because of the possible public health implications,they performed, an extensive characterization of this microorganism and other *Serratia strains*, including determination of their virulence properties for fish and homoeothermic animals.

The present study was undertaken to report the isolation and identi-fication *Serratia marcescens* and all cases of the disease emerging from the investigated fish farms and describe the clinical signs,gross lesion,and histopathology as well as experimental trial for induction of the disease in healthy susceptible fish.In addition,sensitivity test for the isolated bacteria against antimicrobial agents was also achieved.

MATERIALS AND METHODS

(A) History of the investigated fish farms:

Five commercial freshwater fish farms,located in 3 Districts in Kafr El-Sheikh Governorate were investigated during the period 2003-2004 for the cause of remarkable red bands distributed all over the body, exophth-almia, corneal opacity, slow wasting, signs of haemorrhagic septicemia, variable mortalities and they acquired a reddish colour along the lateral line and heed.

All fish farms have been treated previously with various antibiotics, antimycotic and vitamins and fed on a commercial ration *adlibtum* and

used poultry litters. The source of water in all farms originated from soil agriculture drainage tributaries.

Further details on the history of these farms are given elsewhere.

(B) Postmortem examination and specimens collection:

Sick fish as well as freshly dead fish were removed from affected farm ponds and necropsyed using the methods described by ***Plumb and Bowser (1983)*** .

Gross lesions were recorded and specimens from kidney,liver,spleen and eye were collected for bacteriological examination.

(C) Bacteriological Examination:

Specimens from eyes were removed in their entirety as well as from liver,spleen,kidneys and transferred separately to 10 ml volumes of sterile (121˚c /15 min.) 0.9 %(w/v)saline in screw capped Universal bottles with vigorous shaking for 5 minutes to dislodge the microorganisms.Thereafter, 0.1ml volumes were spread over the surface of triplicate plates of tryptic soy agar (TSA;Oxoid) with incubation at 22˚c for 72 hours.The dominant colony types were purified by successive transfer to fresh media ***(Baya et al., 1992)***.

The isolated bacteria were identified by culture morphology, Gram-stain and diagnostic tables according to ***Austin and Austin (1993)*** and ***West and Colwell (1984) and Fouz et al. (1990)***. Biochemical microtest system(API20NE Strip;BioMerieux,Lyon,France)were also used,accding to the manufacturers recommendations (except for an incubation temperature of 25˚c) , on isolates from TSA .

(D) Histopathological examination :

Samples from liver, spleen, kidneys and eyes were fixed in 10 % neutral formalin. The washed soft tissues were dehydrated in different concentrations of alcohol, cleared in xylol and embedded in paraffin.

Sections of 5-6 microns were then cut and stained with haematoxylin and eosin (H&E) stain according to ***Lillie (1984)*** .

(E) Pathogenicity test:

1- For fish :

The *Serratia strains* isolated from O.niloticus were tested for patho-genicity in fingerling *O.niloticus* (4g) and *Mugil cephalus*(7g) maintained at 20 ˚c in freshwater aquaria with aeration. Infectivity trials were condu-cted by intraperitoneal (i.p.) and intramuscular (i.m.) inoculation with bacterial doses ranging from 10^2 to 10^8 (six fish being used per dose) as previously described ***Toranzo et al.(1983)***. Mortality was recorded daily over a one-week period and the lethal dose 50% (LD_{50}) was calculated by the ***Reed and Műench method (1938)***. Surviving fish were sacrificed after a 3-week period in order to attempt the re-isolation of the inoculated strain and, hence, to assess the possible carrier state and also, subjected to post-mortem and histopathological examination.

2- For homoeothermic animals :

To assess the degree of virulence for homoeothermic animals we performed a mouse pathogenicity test following basically the procedure of ***Daily et al. (1981)***. Briefly, between 5 and 10 BALB/C mice (10- 12-weeks-old, 21- 25g) were inoculated intraperitoneally with doses ranging from 10^5 to 10^7 CFU of *Serratia strain*. Mortalities were recorded after 48 hours inoculation and strains displaying an $LD_{50} \leq 10^7$ CFU were considered as virulent ***(Daily et al., 1981 ; Gado and Abd El-Aziz , 2003a)***.

(F) Antibiogramme:

The antibiogramme of *S.marcescens* isolates was investigated against 16 antimicrobial agents using the disc diffusion technique according to ***Criuckshank et al. (1975)***. Such procedure was recommended by the National Committee for ***Clinical Laboratory Standards (1990)***.

RESULTS

(A) Epizootiological features of *S.marcescens* infected farms:

During the period 2003-2004, 5 commercial *O.niloticus* fish farms with history of high mortalities were investigated.

The examined fish from all farms suffered from anorexia, sluggishness, slow wasting, exophthalmia, corneal opacity, variously sized areas of hemorrhage on the skin and they. acquired a reddish colour along the lateral line and head (Figs. 1,2) and sudden variably high mortality of 12.5 - 21.4 %, ten days prior to the investigation (Table 1).

(B) Gross lesion:

Post-mortem examination of freshly dead and killed fish revealed mottled and enlarged liver,gastro-enteritis,spleen enlargement and highly distended gall bladder (Figs. 3,4,5).

(C) Bacteriological examination:

Preliminary tests allowed us to identify the red pigmented colonies, 1-2 mm in diameter, isolated from skin, eyes and various internal organs of all investigated fish farms in a virtual pure culture as belonging to the family Enterobacteriaceae and presumptively to the genus *serratia.* Twenty-eight isolates, which grow on tryptic soy agar revealed motile, Gram-negative, rod shaped organism,could be recovered in a descending frequ-ency order from liver, skin, eyes, intestine, spleen and kidneys (Table 2)**.**

The Biochemical characteristics of the isolated organisms are shown in table (3).key diagnostic features included the oxidase and catalase negative, indole negative, Voges-Proskauer positive, arginine hydrolase negative but lysine and ornithine positive, and gas production from glucose but not H_2S.

The inability of *S.marcescens* to produce acid from arabinose, meliliose, xylose and raffinose allows one to distinguish this species clearly from *S.liquefaciens*(closely related species,***Grimont and Grimont,1984)*** and *S.plymuthica* .

(D) Histopathological findings :

(1) *Eyes:*

Cornea: Showed vacuolated cytoplasm with small peripherally located pyknotic nuclei (Fig. 6) .

Iris : Showed containing high amount of melanin pigment which arranged in irregular areas or in linear manner (Fig. 7).

Sclera: Showed necrobiotic changes and degeneration(nuclear lysis)characterized by massive small pyknotic nuclei (Fig. 8).

Choroidal body : Showed vacuolar degeneration, caseous necrosis, dilated blood vessels and massive aggregation of melanin pigment in the form of spherical bodies (Fig. 9).

(2) *Liver:*

Hepatocytes showed cloudy swelling , necrobiotic changes, thrombosis and focal aggregation of inflammatory cell. Bacteria, presumed to be *S.marcescens*, were associated with the foci of necrosis and randomly scattered in colonies without eliciting a cellular reaction (Fig. 10).

(3) *Spleen:*

Showed focal coagulative necrosis,clumps of vacuoles, severe depletion of lymphoid follicles, severe congestion of blood vessels and haemorrhages (Fig. 11).

(4) *Kidneys:*

Showed haemorrhages,congested blood, cloudy swelling and necrobiotic changes in the renal tubules and coagulative necrosis surrounded by vacuolated tubules.

(E) Experimental results:

(1) *For fish*:

The virulence assays demonstrated that regardless of the inoculation route,the *S.marcescens* isolate was pathogenic for *O.niloticus*(mean LD_{50} of $1x10^5$). Furthermore, in the fish injected intramuscularly the time to death was shorter (1-3 days) than in the fish inoculated intraperitoneally (1-7 days). Some of the dead *O.niloticus* showed strong necrosis of muscular tissues,signs of haemorrhagic septicemia and they acquired a reddish colour along the lateral line and head probably due to the multiplication of the bacterium and the concomitant synthesis of the prodigiosin pigment. Bacterial re-isolation from infected fishes were successful (Table 4).

Histopathological results were nearly similar to those observed in naturally infected fish (Figs. 12-14) .

Fish of the control group remained clinically healthy and showed neither pathological lesions nor *S.marcescens* isolation .

(2) *For mice:*

The virulence assays in mice indicated that *S.marcescens* strain can be considered as virulent since they displayed an LD_{50} of about 10^6 live cell (Table 4) .

(F) Antibiogramme:

Results of sensitivity testing of 28 *S.marcescens* isolates are showed in (Fig. 15) . All tested isolates (100%) showed high sensitivity to enrofloxacin and danofloxacin while 90% of them were sensitive to flumequine and oxolonic acid,80% to sulfamethoxole plus trimethoprim,70% to kitassmycin, 60% to lincospectin,50% to amoxycillin,40% to oxytetracyclin, and 30% to ampicillin.

None of the isolates was susceptible to chloramphenicol, colistin sulfate, streptomycin, gentamycin, erythromycin, and neomycin.

Fig.(1): *O.niloticus* fish naturally infected with *S.marcescens*,showing variously sized areas of haemorrhages on the skin.

Fig. (2): *O.niloticus* fish naturally infected with *S.marcescens*,showing exophthalmia and corneal opacity.

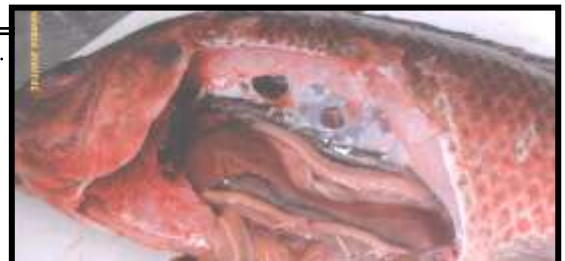

Fig.(3): *O.niloticus* fish naturally infected with *S.marcescens*, showing enlarged mottled liver.

Fig. (4): *O.niloticus* fish naturally infected with *S.marcescens*, showing gastro-enteritis and spleen enlargement.

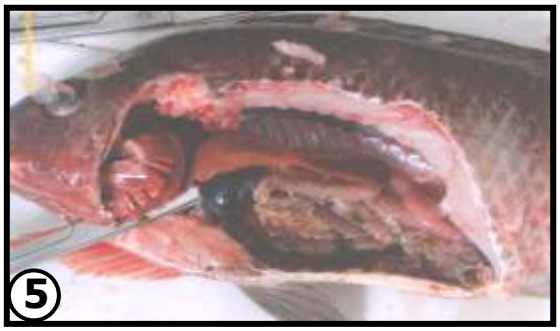

Fig. (5): *O.niloticus* fish naturally infected with *S.marcescens*, showing highly distended gallbladder.

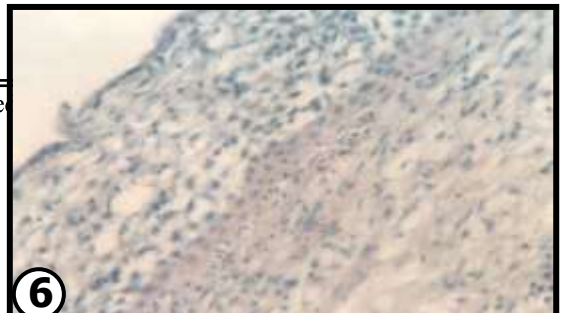

Fig. (6): Sections in eye (Cornea) of *O.niloticus* naturally infected with *S.marcescens*,showing a cellular vacuolated cytoplasm with small peripherally located pyknotic nuclei [H&Ex400].

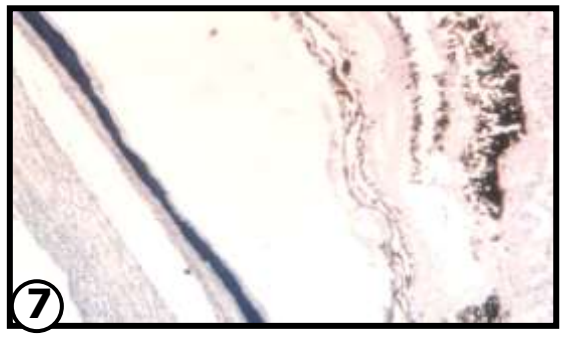

Fig.(7): Sections in eye(Iris)of *O.niloticus* naturally infected with *S.marcescens*, containing high amount of melanin pigment[H&Ex100].

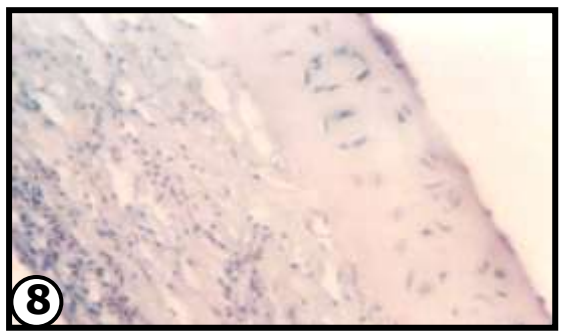

Fig. (8): Sections in eye (Sclera) of *O.niloticus* naturally infected with *S.marcescens*, showing a necrobiotic changes and degeneration with massive small pyknotic nuclei [H&Ex400] .

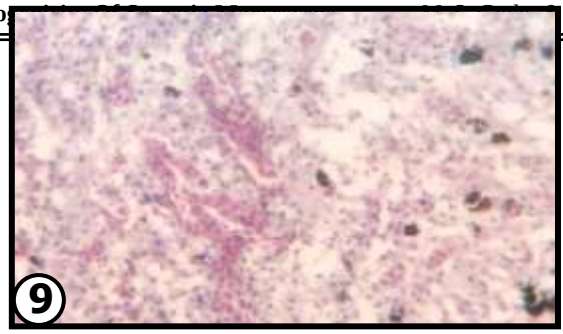

Fig. (9): Sections in eye (Choroidal body) of *O.niloticus* naturally infected with *S.marcescens*, showing a vacuolar degeneration, necrosis, dilated blood vessels and massive aggregation of melanin pigment [H&Ex400].

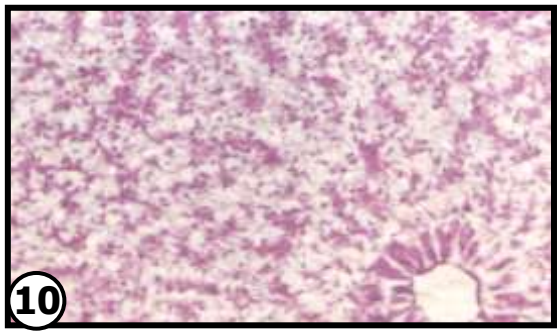

Fig. (10): Sections in liver of *O.niloticus* naturally infected with *S.marcescens*, showing cloudy swelling,necrobiotic changes,haemorrhage and focal aggregation of inflammatory cells [H&Ex400].

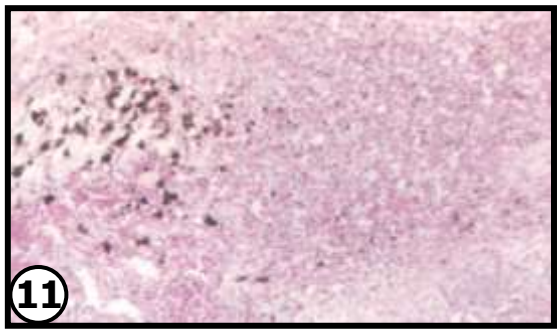

Fig.(11):Sections in spleen of *O.niloticus* naturally infected with *S.marcescens*, showing severe depletion of lymphoid follicles, focal coagulative necrosis and haemorrhages [H&Ex200].

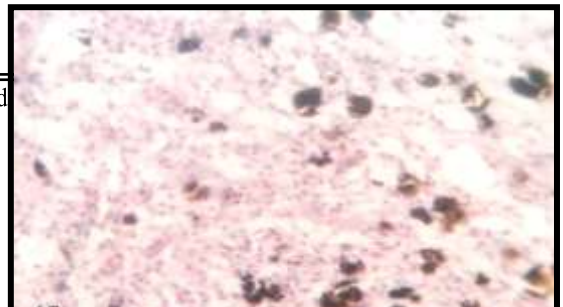

Fig. (12): Sections in eye (Choroidal body) of *O.niloticus* experimentally infected with *S.marces-cens*, showing a massive aggregation of melanin pigment, haemerrhages, necrosis and vacuolar degeneration [H&Ex 400] .

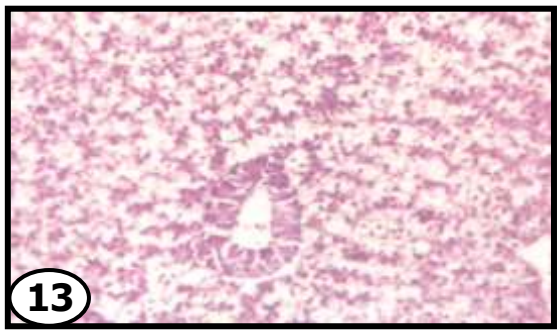

Fig. (13): Sections in liver of *O.niloticus* experimentally infected with *S.marcescens*, showing necrobiotic changes, hydropic degeneration, haemerrhages and focal aggregation of inflammatory cells [H&Ex400] .

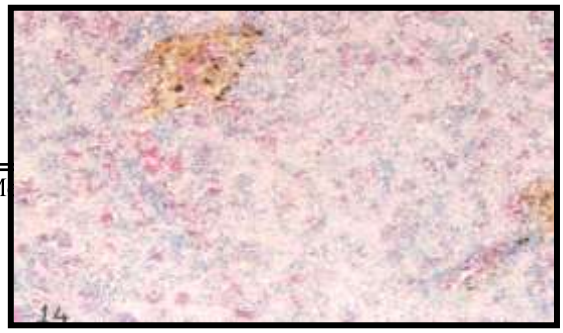

Fig. (14): Sections in spleen of *O.niloticus* experimentally infected with *S.marcescens*,showing severe depletion of lymphoid follicles,degenerative changes in lymphocytes and haemerrhages [H&Ex400].

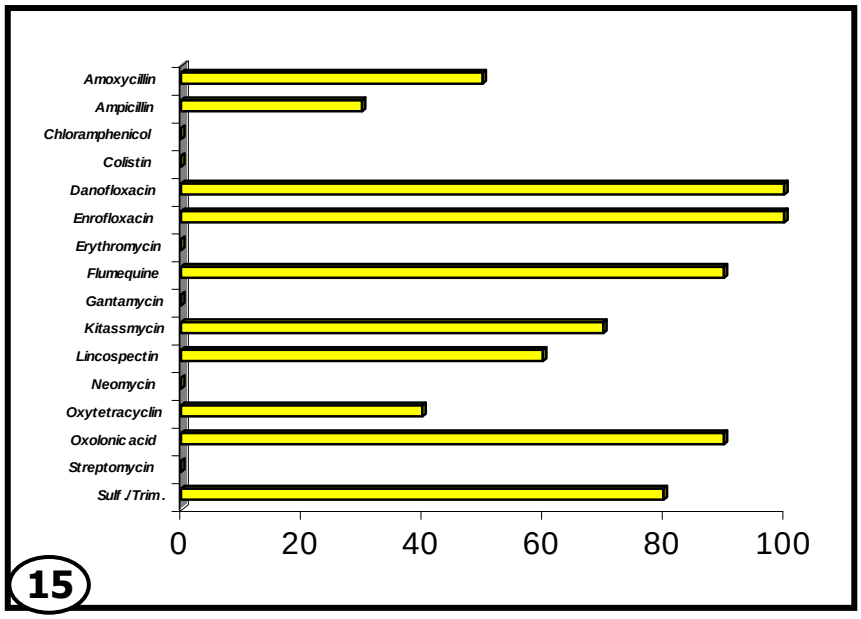

Fig. (15): Results of in-vitro sensitivity of *S.marcescens* isolates(n=28)to different antimicrobial agents.

Table (1): History of examined fish *(O.niloticus)* in the investigated fish farms in Kafr El-Sheikh Governorate.

Code No.	Locality	Farm capacity*/ Fedan	Age / Months	Average body weight / Gram	Mortality %**
1	Hamoul	8	9	210	18.7
2	Hamoul	5	6	155	12.5
3	Reyad	7	6	160	19.8
4	Sedi-Salem	8	5	110	20.9
5	Sedi-Salem	6	4	5	21.4

* Average 11000 fish / Fedan .

** Mortality : Recorded percentage during 10 days prior to the investigation.

No. = Number.

Table (3): Cultural and biochemical characteristics of bacteria isolated from *O. niloticus* fish .

Cultural characters		Isolates of red pigmented colonies on tryptic soy agar plates. N = 28
API* 20 NE	ONPG	+
	ADH	-
	LDC	+
	ODC	+
	CIT	+
	H2S	-
	URE	-
	TDA	-
	IND	-
	VP	+
	GEL	+
	GLU	+
	MAN	+
	INO	+
	SOR	+
	RHA	-
	SAC	+
	MEL	-
	AMY	+
	ARA	-
	OX	-
Catalase		-
The isolated Pathogen		***Serratia marcescens***

*** Code number : 08452. Lot- ch- B. 314962426 .**

ONPG = Beta- galactocidase .
ADH = Arginine hydrolase
LDC = Lysine decarboxylase .
ODC = Ornithine decarboxylase .
CIT = Citrate utilization .
H2S = H2S Production .
URE = Urease .
TDA = Tryptophane desaminase .
SAC = Sucrose [fermentation/ oxidation] .
MEL = Meliliose [fermentation/ oxidation] .
AMY = Amygtalin [fermentation/ oxidation] .
ARA = Arabinose [fermentation/ oxidation] .
OX = Cytochrome oxidase .
IND = Indole production .
VP = Vogus Proskour .
GEL = Gelatinase.
GLU = Glucose [fermentation/ oxidation].
MAN = Manitol[fermentation/ oxidation].
INO = Inositol [fermentation/ oxidation]
SOR = Sorbitol [fermentation/ oxidation].
RHA = Rhamnose [fermentation/ oxidation] .

Table (4) : Virulence for fish and homoiothermic animals of *S.marcescens*.

Groups No.	Animal species	Live cells LD*$_{50}$
1	O.niloticus^{i}	$1.0x10^{5}$
2	M.cephalus^{i}	$5.0x10^{3}$
3	Mice^{i}	$1.6x10^{6}$
4	O.niloticus^{c}	None
5	M.cephalus^{c}	None
6	Mice^{c}	None

I = in fected .

c = control (uninfected) .

*= Mean lethal dose 50% (LD50) was calculated as number of bacteria or Mg protein /g fish needed to kill 50% of inoculated animals .

DISCUSSION

Bacterial diseases remain of major economic and public health importance. We intend to deal with in this study to explore some new possibilities for diagnosis and control of one of the bacterial diseases that continue to cause problems for fish industries of the world. This will be done from point of view of the novel methods rather than from point of view of the disease.

The last 50 years have brought dramatic innovations in all fields of fish medicine.Significant scientific achievements and their organizational realization in the past have been outlined.

A large number of bacterial diseases occur in fish cultures ***(Noga, 1996)***. Among the bacterial diseases encountered in fish, *S.marcescens* infection has been documented as a cause of considerable economic loss to the fish cultures. To date,there are only four documented reports where *Serratia species* have been associated with fish disease ***(Nieto et al.,1990; Llewellyn, 1980; McIntosh and Austin, 1990; Baya et al., 1992)***. It is

motile Gram-negative rod shaped and grow on tryptic soy agar media. The organism was closely resemble to many species of Enterobacteriaceae of clinical interest especially *Yersinia ruckeri* ***(Stevenson and Airdrie, 1984)*** and *Aeromonas veronii* ***(McIntosh and Austin, 1990)*** through it's morphological,colonial,biochemical characters and the septicemic lesions.

The bacterium could be distinguished from other similar pathogens ***(Baya et al.,1992)***.In the present investigation,outbreak of a disease associated with *S.marcescens* infection in 5 farms are described.To our knowledge there have been no reports of the infection or the disease in *Oreochromic niloticus* fish.*S.marcescens* could be isolated from internal skin,eyes and internal organs of the investigated commercial fish farms,which were suffering from anorexia, sluggishness, slow wasting, corneal opacity,haemorrhages and mortality up to 21.4%. Similar finding in *rainbow trout* fish were made by ***Neito et al. (1990)***, in *salmonids* ***(Llewellyn, 1980; McIntosh and Austin, 1990)***. Moreover, we succeeded in isolation of *S.marcescens* from all investigated fish farms, which yielded 28 isolates. Because the organism can cause confusion with other similar bacteria species, the API 20 NE microtest system was a useful tool as a first diagnostic step and considered as a precise method for the identification and differentiation of *S.marcescens* from other bacteria that may cause similar clinicopathologic effects ***(Baya et al., 1992)***.According to the description of ***Grimont and Grimont (1984)***, the biochemical characteristics of the *Serratia strain* isolated in the present study from *O.niloticus* strongly sup-port its assignment to the species *S.marcescens*.

Moreover, most of the clinical signs observed in affected fish farms were similar to signs of infection caused by common bacterial pathogens of *O.niloticus*.The unusual eye opacity and haemorrhagic skin associated with high mortalities which developed in both affected fish farms and experimental infected fish help to distinguish the *S.marcescens* infection from others.

On the other hand, the histopathological lesions in the examined fishes strongly confirned the *S.marcescens* infection and coincided with findings of ***Neito et al. (1990); McIntosh and Austin (1990), and Ullah and Arai (1983)***.

Furthermore, experimental infection of *S.marcescens* in susceptible fishes revealed interesting findings in all response variables measured in this trial when compared with the controls.The strains were highly pathogenic for *O.niloticus*. In addition, the *S.marcescens* isolated from *O.niloticus* was also virulent for *Mugil cephalus*, and was recovered from all the surviving fish3weeks post-inoculation,which indicates that the bacterium can establish a carrier state in this fish species. These findings congruent with those in *rainbow* fish reported by ***Baya et al. (1992)***.

Regarding the virulence assays for homoeothermic animals, *S.marcescens* strains were pathogenic for mice which coincided with the findings of ***Baya et al. (1992)***.

Over many years the scientific disciplines of genetics, husbandry, nutrition,microbiology and immunology were heavily engaged in the creation as well as in the proving of new ideas and concepts of thinking and realisation. Although the origin of *S.marcescens* in the apparently healthy *O.niloticus* is un known, it is probable that the high level of pollution of the area favoured the accumulation of the bacteruim in the fish. In fact, it has been demonstrated***(Buras et al.,1985)***that a high bacterial load in the water stresses the fish immune system and can result in invasion and proliferation in the fish tissues of environmental bacteria.Since the *O.niloticus* fish is a country-wide, we can rule out the risk of the dissemination of the *Serratia* to other geographic zones which is in accordance with ***Baya et al. (1992)***. In addition, the potential pathogenic capability for mammals exhibited by the *O.niloticus* isolate may be of public health concern since *S.marcescens* is a well-recognized opportunistic pathogen causing important human infections.

Regarding the results of *S.marcescens* sensitivity in vitro; all tested isolates showed high sensitivity to danofloxacin and enrofloxacin. The lower incidence of antibiotic sensitivity among isolates of the organism to the other tested preparations might be due to increased resistance which in accordance with the findings of ***Baya et al. (1992)***. In addition, the drug resistance pattern exhibited by this isolates is typical of the majority of the *S.marcescens* strains studied ***(Grimont and Grimont, 1984)***.

The reason for the relatively late appearance of the scientific discipline of epizootiology in the scenario of disease outbreak is largely associated with the complexity of questions and the lack of appropriate tools to study and evaluate multiple inter-relationships.

This is the first report of *S.marcescens* being isolated and identified as a naturally occurring pathogen in *O.niloticus* fish. Because of its similarities with known *O.niloticus* fish pathogens, and its previously unreported occurrence in *O.niloticus* fish,*S.marcescens* effects and prevalence in fish industry are unknown.Clearly,additional biochemical tests are needed when identifying bacteria isolated from *O.niloticus* fish.

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حدوث وظهور إصابة مرضيه بميكروب السراتيا مارسيسينز بمزارع الأسماك في محافظه كفر الشيخ

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أجري استقصاء عن حدوث وباء مرضي غير معتاد في خمسة مزارع بلطي نيلي أهليه تقع في ثلاثة مراكز بمحافظة كفر الشيخ في الفترة بين عامي 2003 – 2004 م. الأسماك المصابة عانت من

فقدان في الشهية، كسل، أنزفة جلدية ذات أحجام مختلفة، عتامة علي قرنية العين ونفوق عالي وصل إلي نسبة 21.4 %. العزل البكتيري من الأسماك المريضة نتج عنة التعرف علي 28 معزولة من ميكروب السراتيا مارسيسينز بواسطة الاختبارات البيوكيميائية.

عند دراسة خواص الضراوة لهذه المعزولات وجد أنها شديدة الضراوة للأسماك عند تراوح الجرعة النصفية للوفاة بين 5 x 310 حتى 1 x 510. وعلاوة علي ذلك تبين أن هذه المعزولات ضارية للفئران عند الجرعة النصفية للوفاة 1.6 x 610.

تم وصف التغيرات الهستوباثولوجية في الأسماك المريضة طبيعياً وكذا المعداه اصطناعيا. كما تم عمل اختبار الحساسية للعترات المعزولة من ميكروب السراتيا مارسيسينز للمضادات الحيوية المختلفة.

هذه الدراسة تعد الأولي من نوعها في تسجيل عدوى ميكروب السراتيا مارسيسينز لأسماك البلطي النيلي كعدوى جديدة في مصر.