National Institute of Ochanography and Fisheries,
Alex. Branch, Egypt.

FLAVOBACTERIUM COLUMNARE INFECTION IN CULTURED OREOCHROMIS NILOTICUS

(With One Table and 11 Figures)

By

SAFENAZ G. MOHAMED and W.D. SALEH*

* Dept. of Microbiology, Fac. of Agriculture, Cairo Univ., Egypt.

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العدوى بميكروب الفلافوباكتيريوم كولومنار فى أسماك البلطى النيلى

صافيناز جمعه محمد أحمد ، وليد ضياء الدين صالح

ميكروب الفلافوباكتيريوم كولومنار المسبب لمرض الكولمنارز، هو من الميكروبات الممرضة للعديد من أنواع الأسماك المختلفة على مستوى العالم. مرض الكولمنارز يصيب الجلد والخياشيم بصفة أساسية. الأمراض البكتيرية فى الأسماك لها بالغ الأثر على الاستزراع السمكى فى مصر. الغرض من إجراء هذا البحث هو تحديد الميكروب المسبب لوباء فى مزرعة أسماك بلطى نيلى خاصة بمحافظة البحيرة فى فصل الصيف لعام ٢٠٠٨م ونتج عنه نفوق للأسماك يقدر بحوالى ١٥%. وقد كانت العلامات الخارجية الظاهرة على الأسماك المصابة بهذا الميكروب فى صورة فقد فى القشور، زيادة إفراز المخاط، وجود بقع بيضاء على ظهر ورأس الأسماك المصابة، بقع نزفيه وقرح على الجلد وتآكل الزعانف. تم إجراء اختبار الحساسية للميكروب وقد وجد أن الأوكسيتتراسيكللين والنيوميسين والاستربتوميسين والكلورامفينكول والكاناميسين واللنكوميسين والايرثيوميسين هى الأدوية التى يمكن الاختيار من بينها.

SUMMARY

Flavobacteruim columnare, causal agent of columnaris disease is pathogenic to many species of freshwater fish throughout the world. The columnaris disease occurred primary on the skin and gills. The Egyptian aquaculture industry is severely impacted by bacterial fish disease. The objective of the present study was to determine the causative agent of an outbreak caused mortality about 15% among cultured *Oreochromis niloticus* in private fish farm in Behera governorate during late summer of 2008. The clinical signs of infected fish were loss of scales, excessive mucus, white spots on the back and head, skin ulcers, hemorrhage and fin erosion. The isolated bacteria developed characteristic rhizoid yellow pigmented colonies that showing swarming. They was gram–negative, filamentous bacteria exhibiting flexing movements Biochemical

characterization proved that the isolated strain could be classified as *F.columnare*. The in vitro antibiotic sensitivity test showed that oxytetracycline, neomycin, streptomycin, chloramphenicol, kanamycin, lincomycin and erythromycin were drug of choices. Pathogenicity studies were performed on *O.niloticus*.

Key words: *Flavobacterium columnare, Oreochromis niloticus, columnaris disease*

INTRODUCTION

Serious losses only occur when pathogen and host are present in an environment which favor the occurrence of the disease. Columnaris disease is example of bacterial disease of fish affecting natural population in rivers or those from farms and hatcheries using freshwater (Austin and Austin, 1987).

If conditions are suitable, a wide variety of fish may be attacked by *F.columnare* and there appear to be no species or family restriction (Anderson and Conroy, 1969). *F.columnare* the causative agent of disease is a pathogen of worldwide important in freshwater fish.

In temperate fish, columnaris disease is recognized by the appearance of grayish white or yellow area of erosion, usually surrounded by a reddish hyperemic zone on the body surface or the gills of fish. When lesions occur around the dorsal fin, they are called saddle – back lesions (Austin and Austin, 1987). Outbreaks of columnaris disease are rarely spontaneous, but are influenced by a combination of environmental (temperature) and other factors stressful to the host, such as high stocking density, high level of ammonia and organic load (Wakabayashi, 1991; Shoemaker *et al.,* 2003; Suomalainen *et al.,* 2006).

In Egyptian aquaculture, many authors recorded columnaris disease in different fish species (Alyan, 1984; Badran and Eissa, 1991) where it induced severe economical losses by increasing the mortality.

The objective of this study was to determine the causes of severe clinical signs and mortality of fish during the outbreak through the isolation and identification of isolated bacteria, as well as its pathogenicity.

MATERIALS and METHODS

The naturally and experimentally infected *Oreochromis niloticus* were submitted to clinical and postmortem examination for any abnormalities as well as bacteriological, mycological and parasitological

examination according to Amlacher (1970). Samples from skin mucus were collected from moribund fish during an outbreak in private fish farm from Elbehera governorate in July 2008. These samples were streaked on Trypticase soya agar (oxoid), and, Shieh agar (Shieh, 1980) supplemented with tobramycine at a concentration of 1ug/ml (Decostere *et al.,* 1997). For the supplemented Shieh agar, a membrane – filtered solution of tobramycin was added after sterilization.

To ensure sufficient moisture content plates with tobramycin were used within 24h. Plates were incubated at 30C and examined after 24, 36 and 48h.

Identification and characterization of bacteria isolated from diseased fish regarding morphology, growth, biochemical and antigenic characteristics were determined according to Decostere *et al.* (1998) and Shoemaker *et al.* (2008).

These characteristics were compared with those of the *Flavobacterium*. *columnare* reference strain NCB2254. Colonies of the bacteria isolated from diseased fish were checked on color, adherence to the agar and rhizoid edges. The suspected colonies were transferred to both Shieh agar and broth. Colour, size, Gram stained, gliding motility of the bacteria and growth aspect after 24h were tested. Growth at different temperatures phenotypic and biochemical traits were carried out using the methods described by Bernarder and Grimont (1989) and compared with the reference strain NCB2254.

The isolated bacteria from the diseased fish was checked for the presence of *F. columnar*e common antigen by slide agglutination using rabbit antiserum against type strain *F.columnare* NCB2254. The test was performed according to the method of Decostere *et al.* (1998).

The sensitivity of the isolated bacteria to different antibiotics was carried out using the dice diffusion technique according to Lennette *et al.* (1980).

Pathogenicity test

Apparently healthy *O.niloticus* (average body weight 60 ± 5g) were kept in 100 L glass aquaria and used for experimental infection.

Prior to experiment 10 fish randomly selected were microbiologically examined and found to be culture negative for the tested isolate (Shoemaker *et al.,* 2003).

Four groups of 15 fish were anesthetized with a solution containing 1g of benzocaine (ethyl aminobenzoate) in 10 ml ethanol. Three groups were intramuscularly inoculated with 0.2 ml PBS containing 10^6CFU of isolated bacteria. A fourth group was

intramuscularly inoculated with 0.2 ml sterile PBS and used as a control (Decostere *et al.,* 1998). All groups were examined clinically 14 days after inoculation.

Mortalities were recorded daily for 14 days and used for culturing to conifirm *F. columnare* presence (Shoemaker *et al.,* 2008).

Histopathological examination were carried out from organs of naturally and experimentally infected fish according to Robert (1978).

RESULTS

Clinical and postmortem changes in naturally infected *Oreochromis niloticus,* the signs of respiratory manifestation including gasping and rapid opercular movements were noticed with poor reflexes especially, escape one. Moribund and freshly dead fish showed discoloration off their back, fin rot, excessive mucus allover the body and superficial hemorrhagic ulcers (Fig.1). The skin around the mouth and opercula was blanched with congested gill lamellae. Slight congestion of liver and kidneys was observed.

The experimentally infected fish revealed the same clinical signs and postmortem lesions. Moreover the site of infection was ulcerated and hemorrhagic (Fig. 2). Some mortalities were occurred in naturally and 20%from experimentally infected *O.niloticus.*

Isolation and identification of *Flavobacterium columnare* strain isolated from diseased fish: Pure yellow – orange flat, rather small with rhizoid edges colonies were detected. The colonies were adherent to the agar showed swarming. The isolated bacteria was Gram. negative, filamentous and exhibiting flexing movement. When grown in flask a filamentary deposit was formed on the base and several thread – like structures were fixed at the side of the flask.

Optimal growth of the isolated bacteria occurred at 25-3C, but failed to grow in the presence of 1% NaCl.

Biochemical characteristics of isolated bacteria as well as from reference strain *F.columnare* were identical. Results are given in (Table 1). Carbohydrate metabolism was found negative. The isolated bacteria from diseased fish was agglutinated by serum against *F.columnare* reference strain. The isolation of bacteria in big quantity were obtained from skin mucus and gills. Moreover, isolation was also done from internal organs but in less quantity. There – isolation of injected *F.columnare* was positive from dead fish. Negative mycological examination was also occurred. Morovere, moderate monogenetic

trematade and Trichodina infestation were found in examined diseased fish.Antibiotic sensitivity.

The in vitro antibiotic test showed that the isolated strain was sensitive to oxetracycline, streptomycin, neomycin, chloramphnicol, Kanamycin linecomycin and erythromycin .Both of colistin and sulfamethoxazole were found to be resistant.

Histopathological alterations

The naturally and experimentally infected fish showed more or less the same histopathological changes. The epidermis showed excessive necrosis and sloughing of the cells with exposure of the underlying dermis which exhibited severe acute inflammatory reaction represented by severe hyperemic and infiltration with leukocytes almost exclusively lymphocytes. Moreover, the underling musculature was involved where some myofibers showed variable degrees of sacroplasmolysis leading to myovacuolation (Fig. 3).

The gill arch showed diffuse severe edema resulting in dispersion of the fibrous connective tissue. Furthermore, there were congestion besides leukocytic and eosinophilic granular cell infiltration. The gill filaments showed slight edema at the base of the secondary lamellae together with moderate epithelia hyperplasia with club shape formation (Fig.4 and 5).

The majority of the liver hepatocytes appeared as signet ring indicating advanced fatty change, particularly the centrilobuar hepatocytes. In addition, other hepatocytes showed individual coagulative necrosis (Fig. 6 and 7).

The posterior kidney showed excessive focal dilatation of the convoluted tubules with eosinophilic hyaline cast. Such tubules became lined with flattened epithelium instead of the cuboidal one. Other tubules suffered from hydropic degeneration and vacuolation of their lining epithelium.Deposition of melanophores as brown patches around few arterioles was noticed (Fig. 8).

The white pulps of the spleen showed slight multifocal lymphoid cell depletion as a result of lymphocytic cell necrosis associated with hyperactivation of melanomacrophage centers.Other areas showed minute foci of coagulative necrosis of the splenic cortex (Fig 9 and 10).

The intestinal villi showed diffuse hyperplasia of the goblet cells (Fig11). Moreover, the submucosa showed severe congestion and edema.

Table 1: Biochemical characteristics of strain isolated from diseased *O.niloticus* and reference strain NCB2254

Oxidase	2/2
Catalase production	2/2
Flexirubin pigment	2/2
No3 production	2/2
H2 Sproduction	2/2
Indol production	0/2
Hydrolysis of	
Starch	0/2
Gelatin	2/2
Lecithin	2/2
Carbohydrate fermentation	
Glucose	0/2
Sucrose	0/2
Lactose	0/2
Maltose	0/2
Mannitol	0/2
Agglutination serum against	
F.columnare	2/2

LEGEND OF FIGURES

Fig.1: Naturally infected *O.niloticus* showing discoloration, excessive mucus and superficial hemorrhagic ulcers.

Fig.2: Experimentally infected *O.niloticus* showing discoloration, hemorrhagic ulcers of the site of injection and hemorrhage of the body.

Fig.3: Skin and underlying musculature of naturally infected *O.niloticus* showing variable degrees of sacroplasmolysis leading to myovaculation. H&E(x400).

Fig.4: Gills of naturally infected *O.niloticus* showing diffuse edema and leukocytic and eosinophilic granular cell infiltration. H&E (X400).

Fig.5: Gills of experimentally infected *O.niloticus* showing club shape formation and moderate epithelia hyperplasia. H&E(x400).

Fig.6: Liver of experimentally infected *O.niloticus* showing heaptocytes appeared as signet ring indication fatty change. H&E(x400).

Fig.7: Liver of naturally infected *O.niloticus* showing coagulative necrosis of the hepatocyts. H&E(X250).

Fig.8: Kidneys of naturally infected *O.niloticus* showing hyaline cast, deposition of melanophores as brown patches around arterioles and hydropic degeneration of tubules. H&E(X400).

Fig.9: Spleen of naturally infected *O.niloticus* showing lymphoid cell depletion and multi foci of coagulate necrosis. H&E(X400).

Fig.10: Spleen of experimentally infected *O.niloticus* showing hyper activation of melanomacrophage centers. H&E(X400).

Fig.11: Intestine of experimentally infected *O.niloticus* showing hyper plasia of the goblet cells and severe congestion and edema of sub-mucosa. H&E(X400).

DISCUSSION

Bacterial diseases are responsible for heavy mortality in both wild and cultured fish (Robert, 1989). *Flavobacteriaum columnare*, the causative agent of columnaris diseases, has been identified as one of the most problematic pathogen in freshwater farming industry (Suomalainen *et al.,* 2005).

Pure bacterial isolate was recovered from *O.niloticus* during the outbreak as well as infestation with trichodina and monogenetic trematode.

Biochemical and morphological characteristics of the isolated strain were identical to those of the reference *F. columnarre* strain. The same characters were reported by (Badran and Eissa 1991; Ellzey *et al.,* 1997; Decostere *et al.,* 1998).

Moreover, the isolated strain from diseased fish was agglutinated by serum against *F.columnare* common antigen, this confirmed that the isolated strain could be identified as *F.columnare* (Decostere *et al.,* 1998).

However, columnaris diseases seems to be a problem among cultured of *O.niloticus* during hot season, probably because of the high temperature favour growth of many *F.columnare* strin (Post 1987; Younis 2000).

The clinical signs of naturally infected fish as well as experimentally one were excessive mucus erosion and necrosis of gill and skin of fish most typically around the dorsal fin, loss of scales hemorrhagic patches on the skin and progressive epidermal ulcer. These signs may be attributed to the adherence of the bacteria to gills and skin and the action of proteolytic enzyme produced by *F.columnare* (Decostere *et al.,* 1999).

The presence of trichodina and monogenea might induce skin and gill abrasion which in turn facilitate the adherence and entrance of *F.columnare* to fish. Many studies have emphasized the possible role of parasites in enhancing infections of fish with secondary pathogens such as bacteria (Pylkko *et al.,* 2006).

The pathways that can lead to such increased susceptibility might be direct, for example when an entrance route for bacteria is created due to epidermal or gills injuries by the parasite (Buchman and Bresciani 1997) or when a parasite act as a vector for a disease (Cusack and Cone, 1986). Also, the recorded parasites may enhanced *F.columnare* infection indirectly via decreased host immuno- competence (Bowers *et al.,* 2000; Bandilla *et al.,* 2006).

Concerning the mortalities in case of naturally and experimentally infected *O.niloticus* may be attributed to the osmotic and electrolyte imbalances as well as multiple changes in fish physiology leading to decrease disease resistance. (Tully and Nolan, 2002; Bandilla *et al.,* 2006).

The successful induction of the disease experimentally leaves no doubt abut the potential pathogencity of isolated *F.columnare*. The fact that infection could occur following the presence in water contaminated with *F.columnare* confirm the invasive character of the organism.

The histopathological changes in organs of infected fish were excessive necrosis and sloughing of the cells with exposure of the underline dermis which exhibited severe acute inflammatory reaction.

The gills showed severe edema and hyperplasia of the secondary lamellae Fatty changes and coagulative necrosis of liver cells, eosinophilic hyaline in convoluted tubules and hyper – activation of goblet cells of intestine were recorded. Moreover, activation of melanomacroplage centers.

These changes were previously reported by Ellzey *et al.* (1997) and Younis (2000). This work raised abut the importance of *F.columnare* as a cause of serious disease among cultured *O.niloticus*. Moreover, the role of the ectoparasite infestation in prevalence of the disease was confirmed.

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