Recent Advances in Diagnosis of Flavobacteriosis Among Some Freshwater Fishes

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Abstract:

Columnaris disease caused by *Flavobacterium columnare* exists worldwide and affects a wide variety of fishes. This study aimed to investigate the isolation and characterization of *Flavobacterium columnare* from naturally infected fishes (*Oreochromis niloticus* and *Clarias gariepinus)* in Ismailia, using conventional biochemical methods and molecular techniques. Bacteriological examination revealed presence of 162 suspected Cytophaga isolates of which only 102 isolates were characterized as *F. columnare* strains according to colony character on Cytophaga agar which appeared yellow or whitish yellow in color, flat irregular with stiletto edge, clear swarming and rhizoid characters. Biochemical characterizations demonstrated that all isolates tested uniformly positive for catalase, gelatin liquefaction, absorption of Congo Red and flexirubin pigment, but they were negative for indol, Methyl red, Vogus Proskauer, and lactose, glucose, saccharose, manitol, arabinose and maltose fermentation. All isolates produced unique and clear PCR bands of the expected size (675 base pair). Consequently, PCR confirmed the positive results of the biochemical reactions of suspected isolated strain and concluded that it is of *F.columnare*.

Keywords: Columnaris disease, *Flavobacterium columnare*, Molecular characterization, PCR, *Oreochromis niloticus* and *Clarias gariepinus*

Introduction:

Flavobacterial diseases were reported firstly by ***Davis (1922).*** These diseases caused by three bacteria within the family Flavobacteriaceae ***(Bernardet and Nakagawa, 2006)***; namely, *Flavobacterium.columnare*, which cause columnaris disease ***(Hawke & Thune, 1992 and Shotts & Starliper, 1999)*** *Flavobacterium psychrophilum*, which cause bacterial cold water disease ***(Nematollahi et al, 2003 and Starliper, 2011)***; and *Flavobacterium branchiophilum*, which cause bacterial gill disease ***(Shotts & Starliper, 1999).***

Flavobacterium columnare, the causative agent of columnaris

disease, is a Gram-negative, non-flagellated bacilli, motile by gliding motility. The ideal temperature for its growth is 25 °C. The bacterium is considered as normal inhabitant in water and fish (***Schneck & Caslake, 2006 and Olivares-Fuster et al, 2007).***

Flavobacterium columnare can infect catfishes of any age, during any season ***(Griffin, 1992).***Columnaris disease is the second most prevalent bacterial disease in channel catfish, about 23% of the total cases of bacterial diseases ***(Hawke and Thune, 1992).***The disease causing economic losses secondary to Enteric septicemia of catfish ***(USDA, 2003).*** In Egypt, it is found that an acute infection with *F. columnare* was the primary cause of mass mortalities among the Nile tilapia (*Oreochromis niloticus*) and Nile catfish (*Clarias gariepinus*) collected from an aquaculture station in Sharkiya Province, during an acute episode of mass kills during the early summer of 2009 ***(Eissa et al, 2010).***

As columnaris disease still faces some of the difficulties regarding the diagnosis, this study was undertaken to investigate the isolation and characterization of *F.columnare* from some naturally infected fishes *O. niloticus and C. gariepinus* in Egypt, using conventional biochemical methods and the recent molecular techniques.

Materials and methods:

Fishes:

A total of 440 grossly affected fishes with columnaris like disease, of which 320 *Oreochromis niloticus* and 120 *Clarias gariepinus* were collected from different localities in Ismailia. These fishes were transferred alive to the Dept. of Fish Diseases and Management, Fac. of Vet. Medicine, Suez Canal Univ., for clinical examinations.

Clinical and postmortem examinations:

The grossly affected fishes with columnaris like disease were subjected for clinical examination according to the methods described by ***Schäperclaus et al (1992).*** Postmortem examination of fishes was done according to ***Conroy and Herman (1981)***. They were sacrificed and examined for the developed lesions in skin, gills, musculature and internal organs.

Bacteriological examination:

Isolation: Naturally infected fishes with columnaris like disease were sacrificed by decapitation, samples were taken aseptically from affected areas of the body surface, mucus of the gills, skin and fins, inoculated into cytophaga broth, incubated at 18° C for 24 hours and then plated on Ordals agar and incubated at 22° C for 72 hrs. Fish surface was disinfected with 70% ethyl alcohol then opened as described by Schäperclaus *et al.* (1992). Samples were taken after sterilization of the exposed surface of the organs from liver, spleen and kidney. All

samples were streaked onto the same plating media. The plates were incubated at 22°C for 72 hrs. Suspected colonies were picked up, streaked on agar slants for purification and further identification. Suspected colonies were picked up from cytophaga agar plates and subcultured on the same medium for purification. A loopful of each pure culture was inoculated into two tubes of semisolid cytophaga agar medium, one of them was used as a stock culture, and the other used for detection of motility. The cultures on slant agar were used for biochemical tests. Final identification of the isolates was based upon the morphological, cultural and biochemical characteristics according to ***Griffin (1992)*** and ***Schaperclaus et al (1992).***

Molecular characterization:

Bacterial isolates:

Seven biochemically identified *F.columna*re isolates, of which four isolates represent the localities from which *Oreochromis niloticus* were collected (F1, F2, F3, F4) and three isolates represent the localities from which *Clarias gariepinus* were collected (F5,F6,F7). All isolates tested for molecular characterization and identification by *Flavobacterium columnare* primers.

DNA extraction

DNA was extracted from bacterial cultures using QIAamp® DNA mini kit (QIAGEN, Germany). Bacterial cells were harvested in a microcentrifuge tube by centrifugation at 5000 × g for 10 min. Cell pellets were re-suspended in 180 µllysis buffer (20 mg/ml lysozym; 20 mMTris-HCl, pH 8.0; 2 mM EDETA; 1.2% Triton) and incubated at 37°C for 30 min. Proteinase K and Buffer AL (20µl) were then added and mixed by vortexing. After 30 min incubation at 56°C, ethanol was added and thoroughly mixed to yield a homogenous solution. DNA was then extracted as per manufacturer's instructions. DNA was extracted from tissue samples by QIAamp® DNA mini kit (QIAGEN, Germany) according to the manufacturer's instructions following the animal tissues protocol.

Polymerase chain reaction procedures

The extracted DNA was amplified using Polymerase Chain Reaction (RT-PCR): using Qiagen one step RT-PCR Kit of 100 reactions. Two specific primers, Forward (598-CAGTGGTGAAATCTGGT-614) and Reverse (1260-GCTCCTACTTGCGTAGT-1276) were used. PCR mixtures contained 200 µM of each deoxynucleotide, 3 mM magnesium chloride, PCR buffer (10 mMTris/HCl, 50 mMKCl, pH 8), 20 pM of each primer, 100 to 125 ng of genomic DNA, and 2.5 U Taq polymerase (Roche Diagnostics, Mannheim, Germany).

Thermal cycling and amplification procedures were done according to the method of Qiagen one step RT-PCR Kit. PCR mixtures contained 1 pg to 500 ng of template DNA, 400 mM of dNTP mix, 0.6mM of each primer (Forwardand Reverse), 2X reaction buffer and 2.5 U of Taq DNA polymerase (Invitrogen, Carlsbad, CA)in 50 ml reaction mixture. Thermal cycling was done in a thermal cycler. The amplification procedure consisted of 30 cycles of amplification. Each cycle consisted of three steps, denaturation of the chromosomal DNA, primer annealing to the bacterial isolate DNA template and primer extension, as follows: 94 ºC for 30 seconds, 45 ºC for 30 seconds and 72 ºC for 2 min. The 30 amplification cycles were preceded by initial denaturation at 94 ºC for 15 min and the final cycle was followed by 8 min primer extension period at 72 ºC after which the final mixture was held at 4 ºC for a period not to exceed 17 h. For long term storage the samples were kept at -20 ºC.

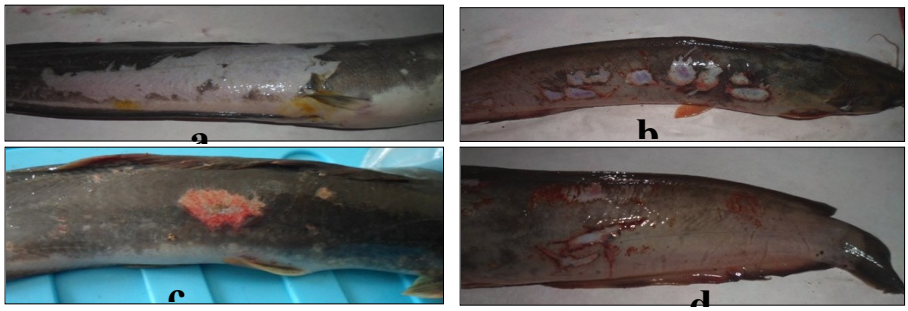
Agarose Gel Electrophoresis:

The PCR products were loaded in agarose gel (50 µl) placed in the electrophoresis chamber and covered with electrolyte solution in 1X TBE with allowing running the PCR product in the gel at 95 V for 30 minute to determine the base pairs of the PCR product (675) bp which could be visualized in the presence of marker (Fermentase) and using Gel documentation system. (Biometra, Germany). Isolates were considered positive when a 675 bp band was detected.

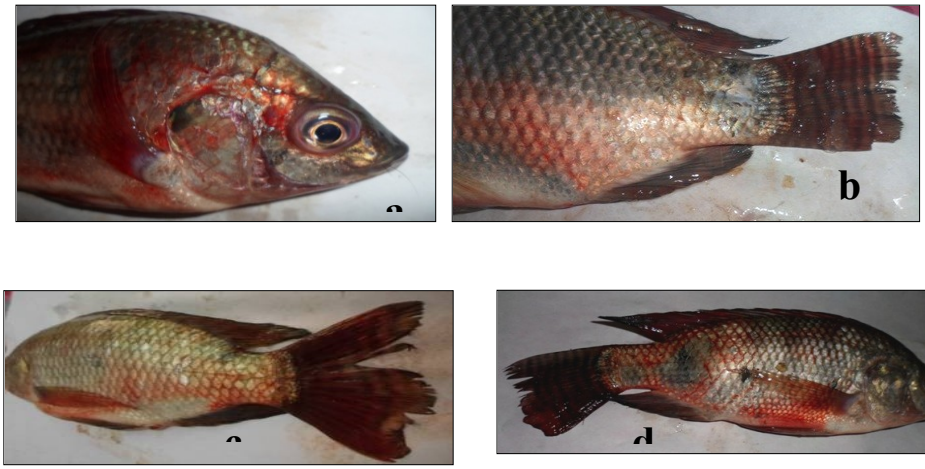
Results:

1. Clinical and postmortem findings of naturally infected fishes:

Naturally infected fishes showed rapid movement of the opercula, floating near the surface with loss of sensation just before death. Some fishes showed excessive mucus, loss of scales in case of *O.niloticus* with erosions and red-grayish patches on head region and abdomen beside the general signs (loss of appetite, dullness, loss of balance, roughness and respiratory distress). In addition to swimming near water surface, gasping and engulfing the atmospheric air were observed. Greyish ulcers were surrounded by red eroded zones, were found at pelvic and anal areas. Hemorrhages were seen at the base of the pectoral fins. The caudal fin was frayed and showed swollen grayish discolored margin. All cases showed necrosis of the caudal fin with presence of grayish spots on the head region were constant findings. Stretched gill covered with pale sticky gills were noted and it was characterized by yellow-brown necrotic tissue at the distal end of the gill filaments with excessive mucus in gill pouches. Some cases showed eye opacity. Also, examination revealed pale internal organs and congested kidneys (Plate 1 and 2).



(Plate 1): *a-Naturally infected Clarias gariepiuns showing loss of shiny appearance with desquamation of the skin (saddle back) lesion***,** *b- showing desquamation of the skin and multiple ulcers, c- showing typical deep ulcers on the skin and musculature,d- showing desquamation of the skin and abrasion.*



(Plate 2): *a-Naturally infected Oreochromis niloticus showing haemorrhagic pectoral fin and necrosis on skin,b- showing loss of scales and erosion of caudal fin,c- showing loss of scales, haemorrhage and necrosis on the caudal fin,d- showing loss of scales, with necrotic area.*

Bacteriolgical examination:

Table (1) summarize the cultural characters and biochemical reactions of suspected *Flavobactrium columnare* strains isolated from both naturally infected fishes. Bacteriological examination revealed presence of 162 suspected Cytophaga isolates of which only 102 isolates were proved as *Flavobacterium columnare* strains.

Primary suspected bacterial cultures isolated from tissues of naturally infected fish on Cytophaga agar showed yellow or whitish yellow coloration, flat irregular with stiletto

edge, clear swarming and rhizoid characters.

Seasonal prevalence of *Flavobacteriosis* among examined fishes (*O.niloticus* and *C. gariepinus*):

Three hundred and twenty *O.niloticus* fish were examined throughout the four seasons of the year where the percentage of fish from which *Flavobacterium columnare* was isolated were zero, 20.9, 33.8 and 12 %, in winter, spring, summer and autumn, respectively as shown in figure (1). On the other hand, a total of 120 *C. gariepinus* fish were examined and the percentage of infection were zero, 26.6, 40 and 8 % in winter, spring, summer and autumn, respectively,as shown in figure (2).

Molecular Identification of *F. columnare* by polymerase chain reaction (PCR).

A pair of 16S RNA gene-based PCR primers, were used for the specific detection of *F.columnare* isolates from tested seven biochemically identified *F. columnare*isolates. All isolates produced unique and clear PCR bands of the expected size (675 base pair) (Fig. 3). Consequently, PCR confirmed the positive results of the biochemical reactions of suspected isolated strain and concluded that it is of *F.columnare*.

Table (1): *Morphological, cultural and Biochemical characteristics of Flavobacterium columnare strains isolated from O. niloticus and C. gariepinus naturally infected with columnaris disease.*

Item	Character
Colonies	Rhizoid Yellowish in colour
Growth on neomycin sulfat and polymyxin B media	+ve
Gram stain	-ve
Morphology	Rods
Binding to Congo red	+ve
Motility	Motile (gliding)
Flexirubin pigment	+ve
Gelatin liquefaction	+ve
Oxidase	+ve
Catalase	+ve
Fermentation of glucose, lactose, saccharose, manitol, maltose and arabinose.	-ve
Methyl red	-ve
Indol, VogusProskauer	-ve

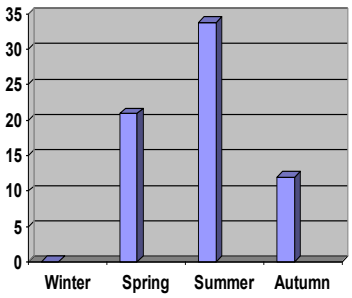


Fig 1 showing Prevalence of *F. columnare* in naturallv

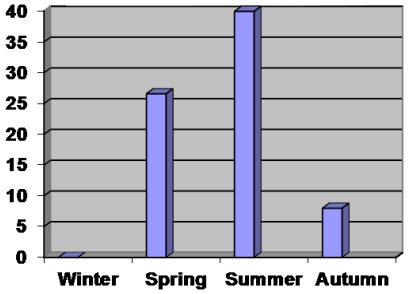


Fig 2 showing

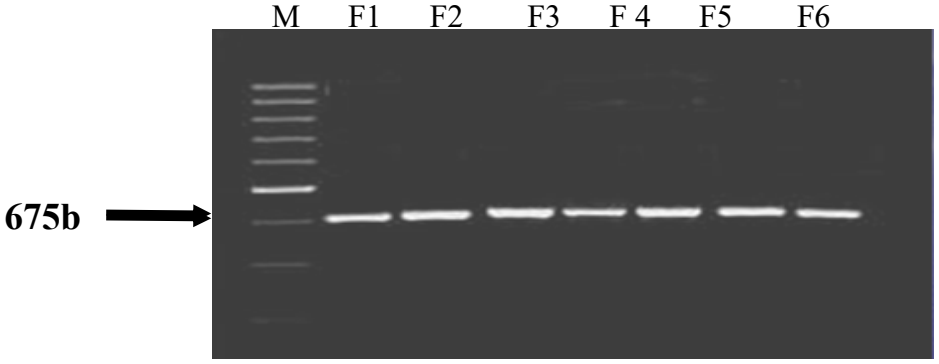


Fig. (3): *Agarose gel electrophoresis of PCR ampilified products of DNA extracted from F. columnare strains using specific primers.*
F1=isolate from*O.niloticus* from cement ponds
F2= isolate from *O.niloticus* from earthen ponds
F3= isolate from*O.niloticus* from Ismailia channels
F5= isolate from *C. gariepinus* from cement ponds
F6= isolate from *C. gariepinus* from earthen ponds
F7= isolate from *C. gariepinus* from Ismailia channel

Discussion:

Columnaris disease, caused by *F. columnare*is is one of the oldest known fish diseases world wide and has been asignificant problem in many warm water fish species for decades.It is one of the most important bacterial diseases of channel catfish, *Ictalurus punctatus*, commercially raised in the US ***(USDA, 2003).***

In this study external lesions of the affected fishes, irrespective of fish species, including erosions, ulcers and fin rot with different degrees. Haemorrhage were seen at the base of the pectoral fins. The caudal fin frayed with grayish discoloured

margin. Necrosis of the caudal fin and presence of grayish spots on the head region were constant findings. These findings are in consistent with those described in many publications (***Morrison et al, 1981; Amend, 1982; Bullock et al, 1986; Amin et al, 1988; Latremouille, 2003; Bernardet & Bowman 2006 ; Eissa et al, 2010 and Declerecq et al, 2013).*** Our results revealed that the seasonal prevalence of columnaris disease in affected fishes reached its maximum at summer season while it was negligible at winter. Therefore, it appears that the columnaris disease is temperature dependent. The relationship between increased *F.columnare* infections and summer in one side and the relationship of increased mortality and severity of infections are temperature dependant have been reported by several authors ***(Chen et al, 1982, Wakabayshi 1991, Suomalainen 2005 and Ahmed et al (2007).*** In addition, predisposing factors that enhance the probability of columnaris disease among cultured fish have been proposed by many investigators including overcrowding, unbalanced nutrition, reduced oxygen contact of water and increase organic load as well as sudden change in pH ***(Sarig, 1976 and Reffat, 2000).*** *F. columnare* can differentiated than other yellow pigmented bacteria by colonies morphology, binding to Congo red, utilization of sugar , degradation of gelatin, production of catalase, hydrogen sulfide and chondroitinase, ***(Griffin, 1992*** and ***Plumb, 1999).***

Our cultural and biochemical characterizations revealed that are specific to *F. columnare* (motility, nitrate reduction, flexirrubin production, absorption of Congo Red, fermentation of glucose and sucrose, desamination of L-tryptophan, gelatin and catalase hydrolysis), demonstrated that all isolates tested uniformly positive for Motility, Oxidase, catalase, gelatin liquefaction, flexirubin pigment and binding to Congo red, but they were negative for indol, Methyl red, Vogus Proskauer, and lactose, glucose, saccharose, manitol, arabinose and maltose. These results agree with ***Decostere et al (1988), Triyanto et al (1999), Triyanto and Wakabayashi (1999), Arias et al (2004), Pilarski et al (2008) and Dong Thanh Ha (2013)*** regardless of the fish species from which they were collected. The absorption of Congo red, which occurred in all isolates, is indicative of galactosamine glucan production by **F. columnare, and Bernadet (1989), Griffin (1992), Decostere et al (1999) and Pilarski et al (2008)** corroborate this fact. All isolates in this study were positive for gelatin hydrolysis, and this is one of the main features of the bacterium *F. columnare.*

Regarding the molecular identification *F.columnare* by PCR produced unique and clear PCR bands corresponded to the 675 bp

internal fragments of the 16S rRNA genes confirmed without doubt that the isolated strains were *F.columnare*. These results were compatible with those was recorded by ***Darwish et al (2004), Eissa et al (2010) and Mohamed & Refat (2011).***

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الملخص العربى

تم اجراء هذه الدراسة لعزل الفلافوفوباكتر كولمنار من اسماك البلطى والقراميط بمحافظة الاسماعيلية باستخدام الطرق التقلدية وكذلك الPCR. بالفحص البكتيرى تم عزل ١٦٢ عترة تبين ان منها ١٠٢ فقط فلافوبكترا كولمنار حسب الخصائص للمستعمرات البكترية التى ظهرت بلون ابيض مصفر متعرجة الجوانب متميزة بشكل التبن.باستخدام الخصائص الكميائية الحيوية وجد ان الميكروب يعطى نتائج ايجابية مع الكتلاز والجيلاتين والكونجو الاحمر وصبغات الفكسوربين وسالب مع الاندول والميثيل الاحمر والكربوهيدرات.جميع العترات تم تأكيد تشخيصها بتقنية الPCRواعطى الحجم المتوقع ٦٧٥ زوج ثنائى.