Original article

Clinical Outbreaks of *Flavobacterium columnare* among *Tilapia* Populations from Lake Qarun, Egypt

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Abstract
During multiple clinical outbreaks that happened between the fall and winter of 2012. A total of 59 fish pathogenic bacteria were retrieved form 150 clinically diseased *Tilapia* species collected from different regions through Lake Qarun. The dermotropic yellow pigmented *F. columnare* have represented 3.4% of the retrieved Flavobacterial isolates. The main clinical findings exhibited by *F. columnare* natural infection were deep ulcerations and tail rot. The lake's poor water quality such as alkaline pH , high levels of un-ionized ammonia, high nitrite concentrations, together with the abrupt rise of water temperature have played a potential role in triggering *F. columnare* infection. To assess the pathogenicity of the retrieved isolates, experimental infection was carried out by intraperitoneal injection of *O. niloticus* with *F. columnare isolates* and mortalities were detected within 10 days. *F. columnare* isolates were sensitive to Danofloxacin, Nalidix acid, Nitrofurantoin and Oxolinic acid while they were resistant to Amoxicillin, Cephalothin, Gentamicin, Lincomycin, Oxytetracycline, Ampicillin, Tetracycline.

Keywords: *Flavobacterium columnare*, *Tilapia*, Lake Qarun, Water quality, Experimental infection

Introduction

Lake Qarun extremely affected by a combination of human activities and climatic changes during the past 5000 years (Flower *et al*. 2006).The lake suffered drastic chemical changes for several decades where it has been used as a general reservoir for agricultural wastewaters drainage as well as fish farms drainage throughout Fayoum province (Authman and Abbas, 2007). Fathi and Flower (2005) reported that the average water temperature of Lake Qarun reached its maximum in August and September (30 °C) and 16 °C as minimum value in February. The regular pH of the lake is an alkaline pH, which has fluctuated between 8.2 in September / October and 9.5 in February.

Tilapias are fast-growing fish which are capable of surviving in poor water conditions and can tolerate a wide range of environmental variables (Amal and Zamri-Saad, 2011). Disease is usually the outcome of an interaction between the host, the pathogen and external stressor(s) (Snieszko, 1974). *F. columnare* infection usually occurs in fish exposed to poor water quality (Tripathi *et al*. 2003). Hansona and Grizzle (1985) induced *Flexibacter columnaris* (recently, *F. columnare*) infections in channel catfish by exposure to 5 mg/L (ppm) nitrite for 7 days. Jeney and Jeney (1995) concluded that *F. columnare* adherence to gill tissues was enhanced by high nitrite concentration (5 mg/L), high organic content (2 g/L) and high temperature (28°C). Tripathi *et al*. (2003) reported that sudden changes in water temperature of 5°C or more pose significant stress, predisposing fish to infection by *F. columnare*. Columnaris disease has also been reported in cold water fish at temperatures ranging from 6 to 12 ºC (Tripathi, 2005). Kubilay *et al*. (2008) demonstrated that mortality level in columnaris infected cultured rainbow trout (*Oncorhynchusmykiss*) fry, reached approximately 30% in a day when water temperature increased to 16°C.

F. columnare is an opportunistic pathogen in which temperature rise above 20 ºC enhances the bacterial pathogenicity and virulence by enhancing bacterial proteolytic enzymes secretion. Thus, increased pathogen virulence together with sharp decrease in dissolved oxygen due to temperature rise will jeopardize the immune system of fish and increases the potential of the ubiquitous bacterial invasion (Eissa *et al*. 2010).

Members of the genus Flavobacterium are widely distributed in soil and water. *Flavobacterium* species induced diseases among fresh water and marine fish (Husien, 1999). The taxonomy of *F. columnare* has been changed several times over the years basis on morpho-chemical criteria. The old taxonomical names of the pathogen included, *Chondrococcus columnaris*, *Cytophaga columnaris* and *Flexibacter columnaris* (Bernardet and Grimont 1989). The most recent name "*Flavobacterium columnare*" was approved by the microbiological community after molecular typing of the worldwide archived strains (Bernardet *et al*. 1996). *F. columare* has been isolated from several fish species worldwide such as Nile tilapia (*O. niloticus*) (Husien, 1994; Husien, 1999); fingerling and adult Nile tilapia in Brazil (Figueiredo *et al*. 2005); Nile tilapia (*O. niloticus*) and Nile catfish (*Clarius gariepinus*) in Lower Egypt (Eissa *et al*. 2010); juvenile rainbow trout in the southern part of Turkey (Kubilay *et al*. 2008), some tropical fish species in Brazil (Pilarski *et al*. 2008).

Kubilay *et al*. (2008) discovered that in wet mount

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Preparation of *F. columnare* showed a slow gliding movement and characteristic column-like masses. *F. columnare* isolates are positive for oxidase, catalase, flexirubin pigment, H_2S production, nitrate reduction and gelatinase tests. Most of isolates grew on Shieh agar supplemented with tobramycin with the development of yellow rhizoid colonies. *F. columnare* do not utilize carbohydrates. Growth can occur at 0.5% NaCl but not in 1% NaCl and no growth occurred at 4°C. *Eissa et al*. (2010) have presented a full morpho-chemical profile for *F. columnare* which included positive catalase; chromo-shift after adding potassium hydroxide 3 % onto the bacterial colonies on Hsu-Shotts agar plates; Congo red binding ; gelatin hydrolysis; growth at 25 ºC, 30 ºC and 37 ºC; growth in the presence of Neomycin sulfate and Polymyxin B sulfate.

Materials and methods

Fish sampling

A total number of 170 apparently healthy and 150 clinically diseased *Tilapias* were collected alive or freshly dead from different localities of Lake Qarun during the period from October 2011 to December 2012 during the course of three emergent outbreaks. The fishes were kept on ice until transferred to the Fish Diseases Research Laboratory for further processing. Clinical and post mortem examination of naturally infected fish were carried out according to the methods described by Stosckopf (1993).

Isolation and purification of *F. columnare*

Skin and gills chunks from examined fish were taken by sterile curved forceps and streaked directly onto Hsu-Shotts agar medium (Bullock *et al*. 1986) supplemented with 0.5 and 1 % NaCl (El-Nasr pharmaceutical chemicals, Egypt). The inoculated plates were incubated at 25ºC for 5-7 days. Presumptive single colonies from the achieved pure cultures were picked and streaked onto different laboratory media: Tryptic soya agar (TSA, Merck, Germany) with 3% NaCl, MacConkey agar (Oxoid, England) and TCBS agar (Oxoid, England).

Colonial and morphological characters

Colonial and morphological characters of *F. columnare* were studied according to Austin and Austin (2012).

Biochemical identification of *F. columnare*

Bacterial isolates were presumptively identified using both cultural characteristics and conventional biochemical tests recommended by Griffin (1992). Biochemical tests include catalase test, adding potassium hydroxide 3 % onto the bacterial colonies on Hsu-Shotts agar plates, Congo red binding (by flooding 24 h cultures with 5 mL of Congo red dye), gelatin hydrolysis, growth on TSA, growth at 25 ºC, 30 ºC and 37 ºC , growth at 0.5 % , 1 %, and 3 % NaCl. Motility was detected by stabbing of the single colony from the pure isolates into semisolid agar. Further identification of *F. columnare* isolates was adopted by API® 20 E and NE; according to the instructions of the BioMérieux Company.

Water analysis

Collection of water samples for physico-chemical parameters testing was conducted according to APHA (1998). Water samples were collected concurrently with fish samples from Shakshouk and Deir-El Birka drains at Lake Qarun basin. Each sample was collected from the mid zone of water source and at a suitable depth in a sterile dry 500 mL glass bottle for chemical examination.

Water temperature was measured by using floating ordinary thermometer (Model Heto T-08- china), while pH was measured using pH meter (Model 25025-Jenway-Germany). Ammonia and nitrite were determined using Colorimeter (Model Jenway 6030-Germany) at wavelength 603 and 545 nm respectively.

Experimental infection

F. columnare was grown on Hsu-Shotts agar medium with 0.5 % and 1% NaCl. The pure cultures were harvested by cotton swab and suspended into sterile saline according to Austin and Austin (2007).

A total of 30 *O. niloticus* with average weight (55±2 g) were used in the experiment. The fish were divided into two groups. First group containing 15 *O. niloticus* were divided into 10 fishes (I/M injected with *F. columnare* at a dosage of 0.5 ml of 10^7 CFU/mL) and 5 fishes (S/C injected with the same dose) according to the method described by Husien (1994). . The second group (control group) comprises 15 *O. niloticus* that were similarly divided into 10 fishes (I/M injected with 0.35 mL saline*)* and 5 fishes (S/C injected with the same dose). The injected fish were kept under observation for 10 days post challenge during which mortalities, clinical signs and PM were recorded on a daily basis. Koch's pathogenicity postulates were fulfilled by re-isolation of the infective pathogen for verification of the specificity of death.

Antibiogram

The disc diffusion method was used as described by Bauer et al. (1966).

Results

Incidence of natural infection of *F.columnare* among examined Tilapia populations

F. columnare prevalence among naturally infected *Tilapia* spp. was (3.4%) among the positive cases of fishes (39) for the different retrieved pathogens. The seasonal prevalence of *F. columnare* was recorded 100% in winter.

Clinical findings in naturally infected fishes

The clinical signs of the *F. columnare* naturally infected fish included deep skin ulcerations, tail rot and hemorrhage at the base of pectoral fins (Figure 1.A and B). On postmortem examination, infected *Tilapias* exhibited pale necrotic gills and enlarged and congested liver.

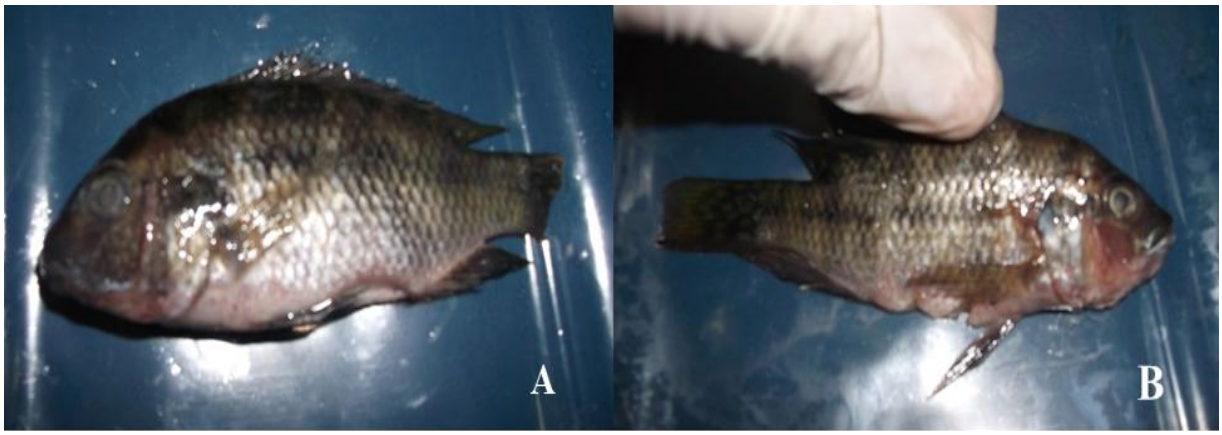

Figure 1: Naturally infected *Tilapia* spp., (A) showing tail rot. (B) showing deep ulceration on the operculum under the eye.

Bacteriological examination

Morpho-chemical characteristics of retrieved isolates

The colonial characters of retrieved isolates on solid media were yellow rhizoid colonies adhering to the Hsu-Shotts agar surface after 5-7 days of incubation (Figure. 2. B). No growth was obtained on TSA 3% NaCl after 7 days of incubation, also no growth on MacConkey agar or TCBS agar. Gram stained smears (Figure. 2. A) from pure colonies revealed the presence of long Gram-negative bacilli. These morphological characteristics were presumptive for *F. columnare*.

Biochemical identification of the retrieved isolates using conventional biochemical tests

F. columnare were identified using a panel of biochemical tests listed in table (1).

Further identification of the retrieved isolates by API® 20 E and NE

The retrieved isolates were further biochemically confirmed using the semi-automated API® 20 E and NE. Isolates identities were confirmed through the API® 20 E and NE software matching system with an accuracy percentage exceeding 98 % .

Intensity of *F. columnare* infection in skin of examined fishes

The intensity of infection (The percentage of *F. columnare* isolation) from skin of naturally infected Tilapias was 100%.

Water quality assessment

The assessed pH of water samples was alkaline while un- ionized ammonia, and Nitrite concentrations exceeded the permissible limits (Table 2).

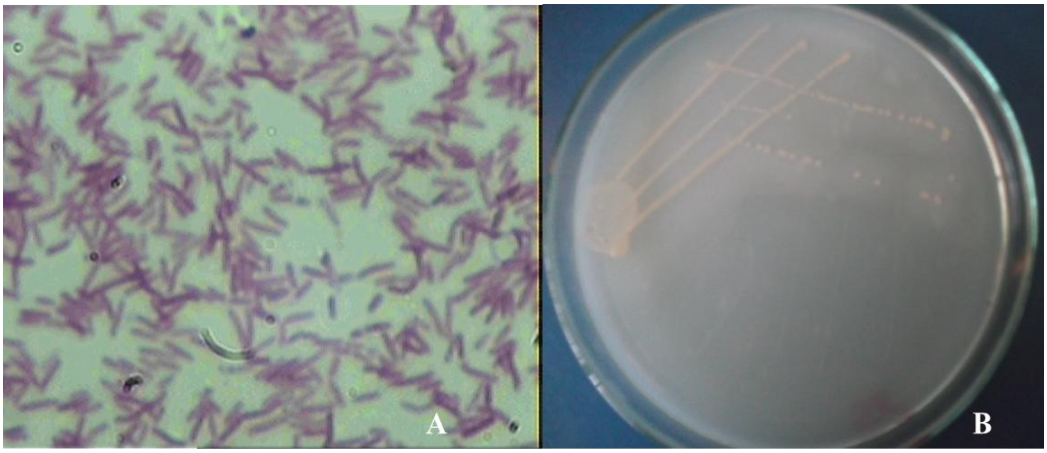

Figure 2: (**A**) Gram- negative long bacilli of *F. columnare*. (**B**)Yellow rhizoid colonies of *F. columnare* adhering to Hsu-Shotts agar surface after 5-7 days of incubation.

Table 1: Biochemical characteristics of *F. columnare*

Biochemical test	**Result**
Oxidase	+ve
Catalase	+ve
Gram stain	-ve long bacilli
KOH 3%	Gm –ve (mucoid)
KOH 20%	Chromoshift to pink-brown colonies
Congo red binding	+ve and at washing with distilled water, no removal of colonies
Indole	-ve
Methyl- Red	-ve
Voges-Proskauer	-ve
Citrate	-ve
Triple Sugar Iron	Alkaline/Alkaline
Hydrogen sulfide production	+ve
Glucose	-ve
Sorbitol	-ve
Sucrose	-ve
Maltose	-ve
Urease	-ve (weak +ve)
Gelatin liquefaction	-ve
O/F test glucose	+/-
Growth at 4^{o}C	Slight growth after 48 hours incubation
Growth at 37^{o}C	No growth after 48 hours incubation
Growth on TSA +3% NaCl	No growth

+ ve = Positive, -ve = Negative, O/F = Oxidation/ Fermentation, +/- = Positive/ Negative

Table 2: Results of water quality assessment

Locality / **Water parameter**	**Shakshouk**	**Deir-El Birka drain**	**References**
Temperature	19 ºC	22 ºC	-
pH	8.51	7.55	-
Un-ionized toxic Ammonia	0.0534 mg/L	0.0126 mg/L	Upper Limit 0.0125 mg/L,(Swann, 1997)
Nitrite	0.02 mg/L	0.02 mg/L	100 µg/L, (Ali, 2002)

Experimental infection

A 60 % of *O. niloticus* I/M injected with *F. columnare* were dead within 6 days post-infection and 40% of those S/C injected were dead within 6 days post infection. It is worthy to mention that mortalities were recorded on a daily basis throughout the 10 days period of the experiment. The clinical signs and post mortem examination of experimentally infected fishes is illustrated in figure (4).

Antibiogram

Antibiograms of the presumptively identified *F. columnare* revealed that all isolates were sensitive to Danofloxacin, Nalidix acid, Nitrofurantoin and Oxolinic acid. All isolates were also resistantto Amoxicillin, Cephalothin, Gentamicin, Lincomycin, Oxytetracycline, Ampicillin, Tetracycline and Clamoxyl, while intermediate resistance was exhibited against Colistinsulphate and Trimethoprim / Sulphamethoxazole (Figure 5).

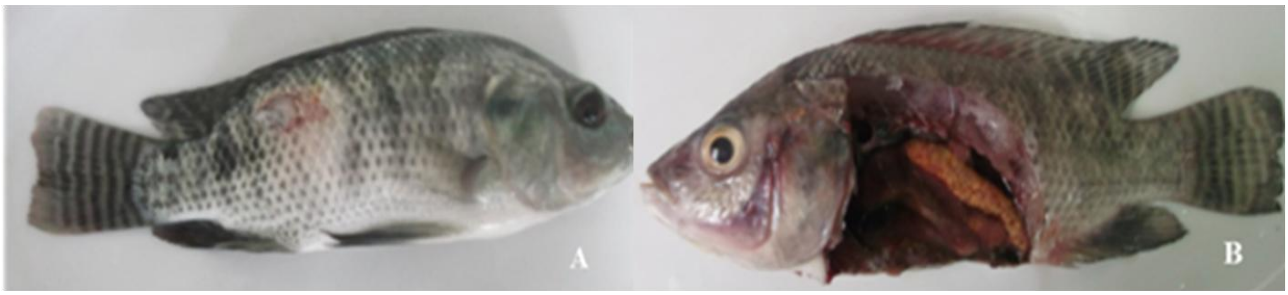

Figure 4: (**A**) *O.niloticus* experimentally infected (S/C) with *F. columnare*, Showed deep hemorrhagic ulceration on the trunk. (**B**) *O. niloticus* experimentally infected (I/M) with *F. columnare* showed hemorrhagic musculuature, enlarged and congested internal organs.

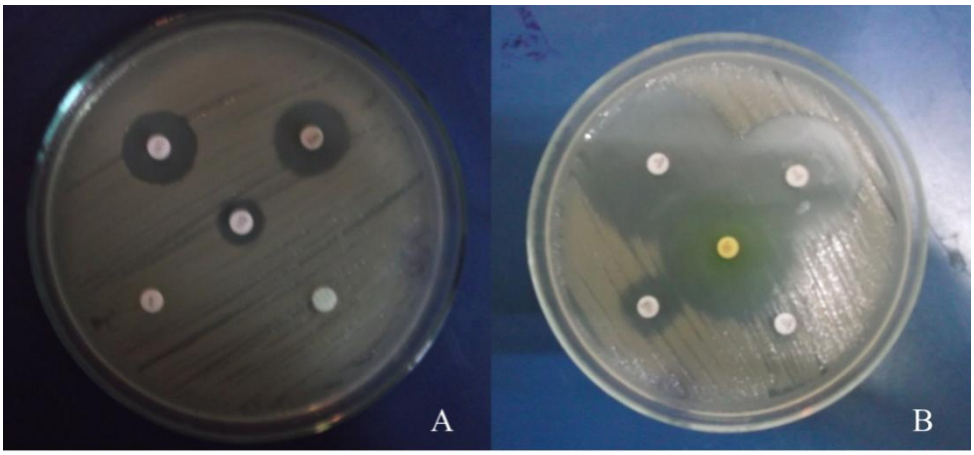

Figure 5: (**A**) *F. columnare* with intermediate resistance to CT and resistant to OT, CN, AMX, MY. (**B**) *F. columnare* sensitive to F, OA, DFX and resistant to AML and AMP.

Discussion

The sharp environmental changes such as sharp fluctuations in temperature, pH and dissolved oxygen are highly incriminated as predisposing factors for initiating *F. columnare* infection (Wakabayashi, 1991 and Tripathi *et al*. 2003). The fluctuations in water temperature above 16 up to 25 ºC then 30 ºC is known to enhance the bacterial pathogenicity and virulence by increasing the bacterial growth rate; increases the adhesion capacity of the bacterium to the fish tissues (Decostere *et al*. 1999); activates the Chondrotin AC lyase (Griffin, 1992) which degrades polysaccharides, particularly those found in cartilaginous connective tissue (Teska, 1993).

The sharp decrease in dissolved oxygen due to temperature rise has suppressed the immune system of fish with increasing the potentials of the ubiquitous bacterial invasions (Amend, 1983; Bullock *et al*. 1986; Suomalainen *et al*. 2005). Further, the increased ammonia levels with consequent rise in water pH are the most possible triggering factors for initiation, establishment and spread of infection (Sniezko, 1974; Holt *et al.* 1975*;* Morrison *et al*. 1981; Bullock *et al*. 1986; Suomalainen *et al*. 2005). Despite the fact that early summer and early autumn are the flaring up seasons for *F. columnare* (Eissa, 2010), yet, the seasonal incidence of *F. columnare* was 100 % in winter season, which is in partial agreement with Peselis (2011) who concluded that Columnaris disease could be enhanced when the fishes are in stressed environments.

The skin ulcerations fin and gill rot can be mainly attributed to Chondroitin AC lyase which is an enzyme produced by *F. columnare*, that has the capability to

degrade polysaccharides, particularly those found in cartilaginous connective tissue (Eissa *et al*. 2010).

The results of morpho-chemical testing of the retrieved isolates were in full agreement with the standard criteria of *F. columnare* described by Austin and Austin (2012), Eissa *et al*. (2010) and Peselis (2011). Controversially, the ability to grow at 4 ºC in which slight growth took place after 48 hours of incubation is against data described by Kubilay at al. (2008) who detected no growth at such temperature. *F. columnare* was 100% isolated from skin which is compatible with the findings of Tripathi *et al*. (2005) who declared that *F. columnare* was readily detected in skin specimens from infected fish. However, the bacterium was infrequently detected in liver, kidney and spleen samples and suggested that Columnaris disease generally occurs as a cutaneous disease that is unassociated with systemic infection.

In respect to experimental infection of *O. niloticus* with *F. columnare*, 60% mortality within 6 days of (I/M) and 40% (S/C) injections were obtained. These results relatively agreed with Husien (1994) who reported 100 % mortalities within 5 days post S/C injection of *O. niloticu*s. Clinical signs and post mortem examination of experimentally infected fishes were almost similar to those of naturally infected fishes. This confirmed that the recorded clinical and PM findings in naturally infected fishes were due to Flavobacteriosis.

Ultimately, the antibiogram results of the confirmed *F. columnare isolates were consistent with similar* data recorded by Husien (1999) who reported complete resistance to Tetracycline and Ampicillin. However, the antibiogram data disagreed with Hawkeab and Thuneab (2011) who mentioned that all *F. columnare* tested strains were susceptible to Terramycin (Oxytetracycline HCl).

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References

Ali MHH (2002). Impact of agricultural and sewage effluents on the Ecosystem of Lake Qarun, Egypt. Ph.D. Thesis, Faculty of Science, Al-Azhar University, Egypt.

Amal MNA and Zamri-Saad M (2011). Streptococcosis in Tilapia (*Oreochromisniloticus*): A Review. *Pertanika J Trop Agric Sci*. 34(2): 195 – 206.

APHA (American Public Health Association) (1998). *Standard Methods for Examination of water and waste water*. 20th edition, APHA, Washington D. C. USA.

Austin B and Austin DA (2007). *Bacterial fish pathogens: Disease of farmed and wild fish* (4th Revised Edition).Springer Praxis Publishing, Chichester, UK.

Authman MMN and Abbas HHH (2007).Accumulation and distribution of Copper and Zinc in both water and some vital tissues of two fish species (*Tilapia zilli* and *Mugil cephalus*) of Lake Qarun, Fayoum Province, Egypt. *Pakistan J Biol Sci*. 10 (13): 2106-2122.

Bauer AW, Kirby WM., Sherris JC, Turck M (1966). Antibiotic susceptibility testing by a standardized single disc method. *Am J Clin Pathol*. 45:493–6.

Bernardet JF and Grimont PAD (1989). Deoxyribonucleic acid relatedness and phenotypic characterization of Flexibacter columnaris sp. nov., nom. rev., *Flexibacter psychrofilus* sp. nov., nom. ver., and *Flexibacter maritimus*. *Int J Syst Bacteriol*. 39 (3): 346-354.

Bernardet JF, Segers P, Vancanneyt M, Berthe M, Kerters K and Vandamme P (1996).Cutting a Gordian Knot: Emended classification and description of the genus *Flavobacterium*, emended description of the family Flavobacteriaceae, and proposal of *Flavobacterium hydatis* nom. nov. (basonym, *Cytophaga aquatilis* Strohl and Tait 1978) . *Int J Syst Bacteriol*. 46 (1):128- 148.

Buller NB (2004). *Bacteria from fish and other aquatic animals: a practical identification manual*. Cambridge: CABI Publishing.

Cruickshank R, Duguid JP, Marmion BP and Swain RHA (1975). *Medical microbiology*. 2nd Volume, 12th Edition, Churchill living stone. Limited Edinburgh, London and New York.

Eissa AE, Zaki MM and Abdel Aziz A (2010). *Flavobacterium columnare* / *Myxobolus tilapiae* concurrent infection in the earthen pond reared Nile tilapia (*Oreochromis niloticus*) during the early summer. *Interdisc Bio Central*. 2 (2): 1-9.

Fathi AA and Flower RJ (2005). Water quality and phytoplankton communities in Lake Qarun (Egypt). *Aquat Sci*. 67: 350- 362.

Figueiredo HCP, Klesius PH, Arias CR, Evans J, Shoemaker CA, Pereira Jr DJ and Peixoto MTD (2005). Isolation and characterization of strains of *Flavobacterium columnare* from Brazil. *J Fish Dis*. 28:199-204.

Flower, RJ, Stickley C, Rose NL, Peglar, S, Fathi, A.A. and Appleby PG (2006).Environmental changes at the desert margin: an assessment of recent paleolimnological records in Lake Qarun, Middle Egypt. *J Paleolimnol*. 35: 1–24.

Griffin BR (1992): A simple procedure for identification of *Cytophaga columnaris*. *J Aquat Anim Hlth*. 4: 63- 66.

Hanson, LA and Grizzle JM (1985).Nitrite-Induced Predisposition of Channel Catfish to Bacterial Diseases. *The Prog Fish Cult*. 47(2): 98-101.

Hawkeab JB and Thuneab RL (2011). Systemic Isolation and Antimicrobial Susceptibility of *Cytophaga columnaris* from Commercially Reared Channel Catfish. *J Aquat Anim Hlth*. 4(2):109-113.

Husien MM (1994). Further Studies on Columnaris disease in Fresh water Fishes. Ph.D.Dissertation. Fac. of Vet.Med., Cairo University.

Husien MM (1999). Natural and Experimental Infection of *Flavobacterium Species* in Freshwater Fish. *Beni-Suef Vet Med J.* 9 (3-A): 311- 326.

Jeney Z and Jeney G (1995). Recent achievements in studies on diseases of common carp (*Cyprinus carpio L.*). *Aquaculture* . 129 (1- 4): 397–420.

Kubilay A, Altun S, Diler Ö and Ekici S (2008). Isolation of *Flavobacterium columnare* from Cultured Rainbow Trout (*Oncorhynchus mykiss*) Fry in Turkey. *Turk J Fish Aquat Sci*. 8:165-169.

Peselis AA (2011). Cytotoxic activity of the bacterial fish pathogen *Flavobacterium columnare* on fish cells. M.Sc. Thesis, University of Rhode Island.

Pilarski F, Rossini AJ and Ceccarelli PS (2008). Isolation and characterization of *Flavobacterium columnare* (Bernardet et al. 2002) from four tropical fish species in Brazil. *Braz J Biol*. 68(2): 409-414.

Snieszko SF (1974). The effects of environmental stress on outbreaks of infectious diseases of fishes. *J Fish Biol*. 6(2), 197-208.

Stosckopf M (1993). Fish Medicine. WB Saunders Company, Philadelphia, Pennsylvania.

Swann L (1997). A Fish Farmer's Guide to Understanding Water Quality.Water Quality. Fact Sheet AS-503.

Tripathi NK, Latimer KS and Rakich PM (2003). Columnaris Disease in Freshwater Fish. *Compendium*. 25(7): 528-536.

Tripathi NK, Latimer KS, Gregory CR, Ritchie BW, Wooley RE and Walker RL (2005). Development and evaluation of an experimental model of cutaneous columnaris disease in koi *Cyprinus carpio*. *J Vet Diagn Invest*. 17:45–54.