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Isolation of *Flavobacterium columnare* from Cultured Rainbow Trout (*Oncorhynchus mykiss*) Fry in Turkey

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Abstract

This study describes columnaris disease from cultured rainbow trout (*Oncorhynchus mykiss*) fry in Turkey in July, 2004. The infection appeared in rainbow trout fry between 5 and 10 g weight in reared raceways where mortality level reached approximately 30% in a day. The appearance of sick fish, besides characteristic skin discolouration and yellow necrotic areas was seen at the tip of the gills. Gram negative long, thin rods were isolated from the anterior kidney, liver and gills of sick fish. *Flavobacterium columnare* was identified by morphology, physiology and biochemical testing using conventional methods and the API 20 E test system.

Key words: Flavobacterium columnare, Oncorhynchus mykiss, columnaris disease, isolation, phenotypic characteristics.

Introduction

Flavobacterium columnare is the etiological agent of columnaris disease, a common bacterial disease affecting the skin and gills of freshwater fish which may cause large mortalities (Frerichs and Roberts, 1989; Noga, 2000). In temperate fish, columnaris disease is recognized by the appearance of greyish white or yellow areas of epithelial erosion, usually surrounded by a reddish hyperemic zone on the body surfaces or gills of fish (Austin and Austin, 1999). *Flavobacterium columnare* has been recognized as a worldwide pathogen of freshwater fish (Bernoth and Körting, 1989; Alvarado *et al*., 1989; Balta and Cagırgan, 1998; Figueiredo *et al*., 2005). It is principally a disease of warm water fish but is well recognized in rainbow trout held at temperatures greater than 14°C, and is associated with stressors such as high stock densities, handling, external injury etc. Juvenile rainbow trout and other salmonids are more susceptible than older fish (Plumb, 1999).

Columnaris disease is primarily an epithelial infection which causes necrotic gill or skin lesions. Infections may become systemic (Thune, 1993; Decostere *et al*., 1999; Noga, 2000). Outbreaks of disease are rarely spontaneous, but are influenced by a combination of water temperature and other factors such as low levels of dissolved oxygen, high levels of ammonia and organic load. Lesions may secondarily be infected by water moulds (Noga, 2000).

In July 2004, heavy mortalities (30%) occurred in rainbow trout (*Oncorhynchus mykiss*) fry cultured in the research unit of the Faculty of Fisheries, Egirdir, Turkey.

The aim of this study was to report the isolation, phenotypic characterization, antimicrobial sensitivity and treatment of *Flavobacterium columnare*, from rainbow trout fry.

Materials and Methods

Sampling

The disease was observed in rainbow trout (*Oncorhynchus mykiss*) fry in July 2004. Increased mortality occurred in three concrete raceways supplied with a mixture of bore-hole water and lake water when water temperature increased to 16°C. Twenty moribund diseased fish ranging from 5-10 g were collected from the raceways. Relevant water quality parameters, namely time dissolved oxygen levels, ammonia and organic load, were measured.

Isolation and Identification

For bacterial isolation, samples were taken from the kidney, liver and necrotic gill lesions. All samples streaked on Cytophaga agar (CA), Shieh agar supplemented with tobramycine at a concentration of 1 µg ml^{-1} (Decostere *et al*., 1997) and Cytophaga agar supplemented with polymyxin B (10 U ml^{-1}) and neomycin (5 µg ml^{-1}) (Plumb, 1999) and glucose-yeast extract-penicillin- streptomycin agar (Austin and Austin, 1989). Plates were incubated at 25°C for 48 and 72 h. Based on the morphology, only one type of colony growth could be determined after 72 hours in internal organs. Colonies of the strain isolated were checked on colour, adherence to the agar and rhizoid edges. Routine test for determination of biochemical characteristics of the bacteria was carried out as described by Sanders and Fryer (1988), Austin and

Austin (1999). A presumptive identification was performed by Gram staining, motility, oxidase activity, catalase production, flexirubin pigment, Congo red absorption, nitrate reduction and H_2S production.

Samples were also plated on Shotts-Watman medium. The salt requirements tolerance on the strain was determined in cytophaga broth containing 0.5% and 1% NaCl.

The API 20E rapid identification system test strips (Biomerieux 20 100 Marcy-l' Etiole, France) were also used for bacteriological diagnosis (Austin and Austin, 1999).

Antimicrobial Sensitivity

Antibiogram tests for the isolate were performed using the disc diffusion technique on Cytophaga Agar. NCCLS guidelines were used for evaluation of the results (NCCLS, 2001). Also, ATB-Vet (Biomerieux 14 289) strip system was used to obtain the antibiotic sensitivity of the isolated strains.

Treatment, potassium permanganate ($KMnO_4$) at 2 mg L^{-1} was used as baths. The treatment with oxytetracycline in feed at 75 mg/kg fish per day for l0 days was present.

Results

The moribund fish were generally aggregated in affected ponds. During the outbreak dissolved oxygen, ammonia and organic load concentrations were 5 mg L^{-1}, 0.0060 mg L^{-1} and 16.4 mg L^{-1} respectively.

Characteristic clinical signs included skin discoloration, yellow necrotic gill lesions at the tips of the lamellae (Figure 1), and dorsal fin damage. Internal symptoms included acidic fluid.

Colonies developed from the internal organs at 25°C in 48 and 72 hours resulted in growth of usually pale yellow flat, rather small and had rhizoid edges and tended to adhere to the cytophaga agar. The isolated bacteria were Gram negative slender and rather long bacilli. In wet preparation, the bacteria showed a slow gliding movement and characteristic of column-like masses (Figure 2).

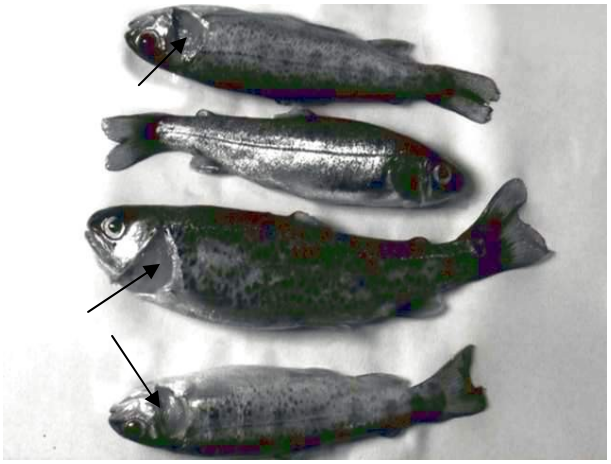

Figure 1. Gill lesions of rainbow trout infected by *F. columnare*.

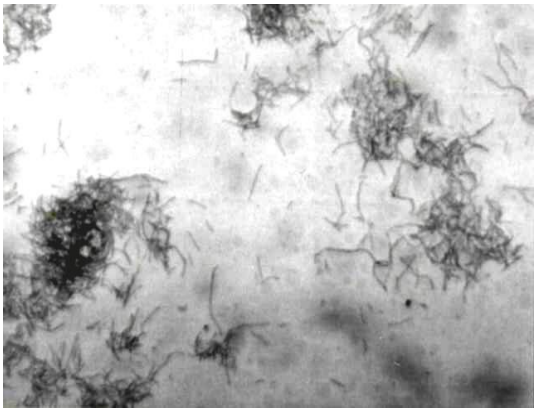

Figure 2. Aggregations of, long, thin rods typical of *F. Columnare* x1000.

The biochemical and physiological characteristics of the bacteria are given in Table 1. Oxidase, catalase, flexirubin pigment, H_2S production, nitrat reduction, congo red absorption and hydrolysis of gelatine tests were positive. The isolated strain grew on Shieh agar supplemented with tobramycin as well as CA with polymyxin and neomycin as yellow rhizoid colonies and produced gelatinase. Carbohydrates were not metabolised. Growth occurred with 0.5% NaCl but not 1% NaCl and no growth occurred at 4°C. According to these morphological and biochemical characteristics of strain isolated from rainbow trout fry, it was identified as *Flavobacterium columnare*.

Saprolegnia spp. was also isolated and identified from gill lesions, but was considered to be a secondary opportunistic infection.

The mortality was reduced following $KMnO_4$ and oxytetracycline treatment. Antibiotic sensitivity tests showed that the *F. columnare* isolate was sensitive to oxytetracycline (30 µg), chloramphenicol (30 µg), furazolidone (100 µg), nitrofurontoin (300 mcg), erythromycin (15 µg) and streptomycine (10 µg) (Table 2). When tested with the ATB-Vet strips the strain was sensitive to chloramphenicol, oxytetracycline, furazolidone, nitrofurantoin, erythromycin, streptomycin, penicillin, amoxicillin, amox-clav.acid, cephalothin, spectinomycin, gentamicin, apramycin, tetracyclin, doxycyclin, lincomycin, tylosin, cotrimoxazol, sulfamethizol, flumequin, oxolinic acid, enrofloxacin, nitrofurantoin, fusidic ac, rifampicin and metronidazol (Table 3).

Discussion

Columnaris has been reported as a mortality factor in several species of cultured fish a systemic myxobacterial infection which caused heavy mortalities; and it was observed in rainbow trout fingerlings (0.7 to 10 g) when water temperature is ranging from 5 to 15°C (Rintamaki-Kinnunen *et al*., 1997). Although there was no mortality *F. columnare* was isolated from eel in commercial intensive warm water recirculation aquaculture unit at 25±2°C water temperature (Alvarado *et al*., 1989) and skin lesions in 3 years old tench respectively (Bernoth and Körting, 1989). Cytophaga family was isolated from external lesions in cultured carp, producing ulsers surronded by a red zone (Bootsma and Clarx, 1976). Also *F. columnare* was observed in intensively cultured walleyes and hybrid walleyes and caused fin erosion (Clayton *et al*., 1998). The symptoms of the

Table 1. Phenotypic characteristics of isolated *Flavobacterium columnare*

Characteristics	Response
Colony pigment	Light yellow
Colony shape on CA	Flat, spreading Rhizoidal edges
Gram Stain	-
Morphology	Thin, long rods
Gliding Motility	+
Oxidation-Fermentation(O-F)	-
Sensitivity to O/129	+
Production of:	
Catalase	+
Oxidase	+
Flexirubin-type pigment	+
Nitrate reduction	+
Congo red adsorption	+
*(ONPG)-Beta-Galactosidase	-
*(ADH)-Arginine dihydrolase	-
*(LDC)lysine decarboxylase	-
*(ODC) Ornithine decarboxylase	-
*(URE) Urease production	-
*(TDA) tryptophane deaminase	-
*(IND) Indol	-
*(VP)Voges Proskauer reaction	-
*H_2S production	+
Degradation of:	
Casein	+
*(GEL)Gelatine	+
Starch	-

Characteristics	Response
Utilisation of:	
*(CIT)Citrate	-
Acid production from:	
*(GLU)Glucose	-
*(MAN)Mannitol	-
*(INO)İnositol	-
Lactose	-
*(SOR)Sorbitol	-
*(RHA)Rhamnose	-
*(SAC)Sucrose	-
*(MEL)Melibiose	-
Mannose	-
*(ARA)Arabinose	-
*(AMY)Amygdalin	-
Growth on:	
Shieh medium supp. tobramycin	+
CA supp. Polymyxin and neomycin	+
Trypticase soy agar	-
Shotts_Waltman medium	-
0% NaCl	+
0.5% NaCl	+
1% NaCl	-
Growth at:	
4°C	-
30°C	+

(+): positive reaction; (-): negative reaction)

Table 2. Sensitivity of isolated *Flavobacterium columnare* to antibiotics

Antibiotics	Zone size (mm)	Sensitivity
Chloramphenicol (30 µg)	34	S
Oxytetracycline (30 µg)	40	S
Furazolidone (100 µg)	36	S
Nitrofurantoin (300 mcg)	40	S
Erythromycin (15 µg)	35	S
Streptomycin (10 mcg)	16	S
Tobramycin (10 mcg)	0	R
Oxalicilin (1 µg)	0	R
Cotrimoxzole (25 µg)	15	R
Kanamycine (10 µg)	10	R

S: sensitive, R: resistant

Table 3. ATB-VET (14 289) Kit results for *Flavobacterium columnare*

Antibiotics	(mg/l)	Sensitivity	Antibiotics	(mg/l)	Sensitivity
Penicillin	0,25	S	Doxycyclin	4	S
Amoxicillin	4	S	Erythromycin	1	S
Amox-clav.ac	2	S	Lincomycin	2	S
Oxacillin	2	R	Pristinamycin	2	R
Cephalothin	8	S	Tylosin	2	S
Cefoperazon	4	R	Colistin	4	R
Streptomycin	8	S	Cotrimoxazol	2/38	S
Spectinomycin	64	S	Sulfamethizol	100	S
Kanamycin	8	S	Flumequin	4	S
Gentamicin	4	S	Oxolinic ac.	2	S
Apramycin	16	S	Enrofloxacin	0,5	S
Chloramphenicol	8	S	Nitrofurantoin	25	S
Tetracyclin	4	S	Fusidic ac.	2	S
Rifampicin	4	S	Metronidazol	4	S

S: sensitive, R: resistant

infected fish are similar to those observed by Durborow *et al*. (1998) in channel catfish with *F. columnare*.

In present study, *F. columnare* isolates were tested to the API 20E and produced consistent results, indicating the API systems ability to identify *F. columnare* isolates by biochemical phenotype. Hydrolysis of gelatine and H_2S production tests were positive in the API 20E test system. Carbohydrates were not metabolized in the identification system. The results were similar to the reports of Austin and Austin (1999). Farmer (2004), reported that ten *F. columnare* isolates tested produced consistent results in the API NE and API ZYM systems, indicating the API systems have the ability to consistently identify *F. columnare* isolates by biochemical testing.

Mortality was reduced following $KMnO_4$ and oxytetracycline treatment. These results are similar with the Thomas-Jinu and Goodwin (2004b) reports.

According to these morphological and biochemical characteristics of strain isolated from rainbow trout fry, it was identified as *Flavobacterium columnare* according to Holt *et al*. (1994), Decostere *et al*. (1997), Plumb (1999), Austin and Astin (1999) and Thomas-Jinu and Goodwin (2004a)

Characteristic for this disease is that the outbreaks are related to stress (high temperature, low levels of dissolved oxygen and high levels of ammonia and organic load) and mortality of fish peak over the course of a few days. Available information suggest that in the aquatic environment, *F. columnare* should be considered as opportunistic bacteria with potential to become pathogenic for fish under stressful conditions.

In conclusion, this study represented the systemic *F. columnaris* infection isolated from juvenile rainbow trout in the southern part of Turkey. Although *Saprolegnia* spp. was also isolated from gill lesions, it was considered to be a secondary opportunistic infection.

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