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First Isolation of a *Flavobacterium johnsoniae* like Bacteria from Cultured Russian Sturgeon in Turkey

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Abstract: The aim of this study was to identify the causative agent responsible for low losses in cultured Russian sturgeon (*Acipencer gueldenstaedtii*) in Turkey. Two outbreaks occurred in the autumn of 2007 and 2008 after heavy rainfall accompanied by a sudden change in water temperature and increased suspended solids. The sturgeons displayed ulcerations, haemorrhage and superficial skin erosions especially on the ventral side including the pectoral and pelvic fins as reported in other studies. Affected fish were lethargic displayed excessive mucus secretion with skin lesions appearing as pale grey spots. Phenotypical characterizations were done according to standard protocols and supplemented with commercial APIZYM, API 20E and API 20NE kits. Sequencing of 16S rDNA PCR products were performed to genetically confirm the identity of the isolated organism.

Key words: *Flavobacterium johnsoniae*, Russian sturgeon, *acipencer gueldenstaedtii*, bacterial infection, 16S rDNA, Turkey

INTRODUCTION

Turkish aquaculture has undergone a rapid development and expansion over the last two decades. To this end, Russian sturgeons (*Acipenser gueldensteadtii*) have been maintained at Sapanca Inland Water Fish Culture Research and Applied Station (Istanbul University, Fisheries Faculty) for restock management and as a basis for possible future commercial sturgeon farming in Turkey.

Although sturgeons are relatively resilient to disease, several bacterial, viral and parasitic diseases have been reported worldwide including Columnaris disease, Motile Aeromonas Septicemia, Yersiniosis, Epitheliocystis, Cytophaga-like infection and White Sturgeon Adenovirus Disease, White Sturgeon Herpesvirus Disease, White Sturgeon Iridovirus Disease, White Sturgeon Papova-like Virus Disease (Adkinson *et al*., 1998; Bauer *et al*., 2002; Mokhayer and Masouleh, 2005).

Flavobacterium johnsoniae (previously *Cytophaga johnsonae*, *Flexibacterium aurantiacus*) is the causative agent of false columnaris disease, gill disease, skin disease of barramundi, salmonids, koi and sturgeon in Europe, Australia, France, UK and USA. *F. johnsoniae* was long known as a common soil organism and frequently recognized in external lesions of different fish species world wide (Carson *et al*., 1993; Bernardet and Bowman, 2006; Flemming *et al*., 2007). Outbreaks in fish farms occur under intensive farming conditions in association with increases in suspended solids in the water and sudden falling in water temperature. *F. johnsoniae* as well as other *Cytophaga* like bacteria have been associated with fish disease and have also been detected in surrounding water during disease outbreaks (Bernardet and Bowman, 2006).

The aim of this study was to identify the causative agent responsible for the low losses affecting cultured Russian sturgeon (*Acipencer gueldenstaedtii*) in Turkey under similar circumstances.

MATERIALS AND METHODS

Case history: Fertilized eggs of Russian sturgeon (*Acipenser gueldensteadtii*) were brought from the Krasnodar Research Institute of Fisheries in southern Russia to the Sapanca Inland Water Fish Culture Research and Applied Station in 2001 (Istanbul University, Fisheries Faculty). The outbreaks occurred in the autumn of 2007 and 2008 after heavy rainfall followed by a sudden change in water temperature and high turbidity. The events had been observed in previous

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years but due to lack of funding no investigation into the problem could then be initiated. The cumulative mortality was approximately 1-2% and the 5 sampled fish exhibiting clinical signs were about 6 or 7 years old.

Isolation and characterization of the causative agent: Water samples collected in sterile tubes and fish samples taken from internal organs (liver, kidney, spleen) of the five diseased sturgeons using sterile loops and aseptic techniques were directly streaked onto TSA agar plates and incubated for 48-72 h at 22°C. Phenotypical characterizations were done according to standard protocols and supplemented with commercial APIZYM, API 20E and API 20NE kits (BioMerieux, France) according to manufacturer's instructions (Whitman and MacNair, 2004; Austin and Austin, 2007). In addition, the identity of the causative agent was investigated genetically by sequencing 16S rDNA gene PCR products.

Genomic DNA extraction: Three isolates were inoculated into TYES and incubated over night at 23°C in a shaking incubator. Genomic DNA from the 3 isolates were extracted by the High Pure PCR Cleanup Micro Kit (Roche) and used as template for PCR.

PCR and 16S rRNA gene sequencing: Genomic target DNA was amplified using the 16S rRNA gene genus spesificprimersets;F1 5'-AGAGTTTGATCITGGCTCAG-3' andR5 5'-ACGGITACCTTGTTACGACTT-3' andF3 5'-GCCAGCAGCCGCGGTAATAC-3' and R5 5'-ACGGIT ACCTTGTTACGACTT-3' (Flemming *et al*., 2007). PCR reaction mixtures included approximately 100 ng template DNA, 10 pmol of each Primer and 25 μL 2×PCR master mix (Fermentas). Amplification was performed using the Biometra thermal cycler and the following parameters: 94°C for 5 min followed by 35 amplification cycles including denaturation at 94°C for 30 sec, primer annealing at 55°C for 1 min and extension at 72°C for 1 min and a final extension step of 72°C for 8 min (Flemming *et al*., 2007). PCR products were visualized on a UV transluminator and size estimated against GeneRuler 50 bp DNA Ladder (Fermentas SM0371) after Etbr staining and gel electrophoresis (1.2% TAE agarose gels, 75 Volt and 40 min). PCR products were cleaned and sequenced by REFGEN (Ankara, Turkey) using the said primers. Sequence editing and analysis was performed using Bioedit v7.0.9. (Hall, 2007) and BLASTN 2.2.20 algorithm (Zhang *et al*., 2000).

RESULTS AND DISCUSSION

The diseased sturgeons were lethargic had ventral ulcerations, hemorrhages and superficial skin erosions

Fig. 1: Haemorrhage and superficial erosion on the skin, especially on the ventral side of body including the pectoral and pelvic fins and the ventral area of the rostrum

especially on the ventral side including the pectoral and pelvic fins as reported in previous studies (Carson *et al*., 1993; Mokhayer and Masouleh, 2005; Austin and Austin, 2007) (Fig. 1). In addition, we observed skin lesions appearing as pale grey spots and excessive mucus secretion.

On examination of primary TSA agar plates only one type colonies were seen. The bacteria from the liver and kidney of diseased sturgeons were long rod shaped, Gram negative, exhibiting gliding motility, cytochrome oxidase positive, ONPG hydrolysis positive and fermentative. Colonies were yellow with flexirubin pigments spreading with filamentous margins and flat on Modified Anacker and Ordal Agar (MAOA). Other physiological and biochemical characteristics are shown in Table 1. The presented isolate characteristics and observed external findings on diseased fish are in agreement with those reported by other researchers (Carson *et al*., 1993; Tamaki *et al*., 2003; Bernardet and Bowman, 2006; Buller, 2004; Austin and Austin, 2007) which led to a presumptive *Flavobacterium johnsoniae* identification. The identification profile in API 20E system was 1203004. Although, *F. johnsoniae* is not included in the API database, this identification code has been reported as a typical profile for *F. johnsoniae* strains (Buller, 2004; Bernardet and Bowman, 2006).

F. johnsoniae as well as other *Cytophaga* like bacteria have been associated with fish disease and have also been detected in surrounding water in the presence of disease outbreaks (Bernardet and Bowman, 2006). Simultaneously, we also identified *F. johnsoniae* like bacteria in water and diseased rainbow trout (~2-3 g) at the same location. These opportunistic bacteria can be pathogen under special condition (such as a sudden change in water temperature or other negative environmental factors, stress caused by handling) as

Table 1: The morphological and biochemical characteristics of the isolates

Characteristics	Results
Gram stain	-
Cell morphology	Long rod
Gliding motility	+
Cytochrome oxidase	+
O/F test	O
O/129 10 μg	R
O/129 150 μg	S
Indole	-
Growth in 0% NaCl	+
Growth in 0.5% NaCl	+
Growth in 1% NaCl	+
Growth in 22°C	+
Growth in 37°C	-
Degradation of starch	+
Aesculin	+
Flexirubin type pigment	+
Tween 80	+
β-galactosidase	+
Arginine dihyrolase	-
Lysine decarboxylase	-
Ornithine decarboxylase	-
Citrate	+
Production of hydrogen sulphide	-
Urease	-
Tryptophane deaminase	-
Voges-prokauer	+
Gelatin	+
Glucose	-
Mannitol	-
Inositol	-
Sorbitol	-
Rhamnose	-
Sucrose	-
Melibiose	-
Amygdalin	-
Arabinose	-
Growth on trypticase soy agar	+
Nutrient agar	+
Nutrient agar (1% SDS)	-
API 20E	1203004
API 20NE	1472305
APIZYM	-+-+-++
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+: Positive result; -: Negative result

other researchers have reported (Carson *et al*., 1993; Soltani *et al*., 1994; Bernardet and Bowman, 2006). The assembled contiguous 1331 bp sequence showed 99.0% 16S rRNA sequence similarity to *Flavobacterium* sp. strain Tibet S721 (DQ177492). The highest 99.4% sequence similarity found however following a short 22 bp region in the 5 end of the gene coding sequence was to *F. johnsoniae* strain 188 (EU730945). A 14 bp mismatch there reduced the overall sequence similarity to 98.4% similar to that found throughout near ful length *F. saccharophilum*, *F. hydatis* and *F. hercynium* gene coding sequences.

The sequence can not be chimerical as the template DNA was extracted from a pure culture isolate. Biochemical results also point toward identification of *F. johnsoniae*. The three isolates could be differentiated from *F. saccharophilum* by production of cytochrome oxidase and H_2S, from *F. hydatis* by production of flexirubin-type pigment and from *F. hercynium* by production of N-acetyl-β-glucosaminidase and no production of a-glucosidase (Bernardet and Bowman, 2006; Cousin *et al*., 2007). Taken together, Flavobacterium johnsoniae-like bacteria is the likely cause of cultured sturgeon mortalities in Turkey.

CONCLUSION

In this study, the cause of death in the sturgeons in question was associated with a *Flavobacterium johnsoniae* like bacteria infection. These opportunistic bacteria can be pathogenic under special conditions (such as following handling, a sudden change in water temperature or other environmental stressors).

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