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ISSN 0792 - 156X

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PUBLISHER:

Israeli Journal of Aquaculture - BAMIGDEH -
Kibbutz Ein Hamifratz, Mobile Post 25210,
ISRAEL

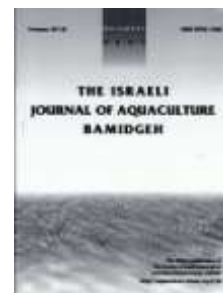
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Isolation of *Shewanella putrefaciens* from Goldfish (*Carassius auratus auratus*)

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(Received 7.2.13, Accepted 3.4.13)

Key words: *Shewanella putrefaciens*, PCR, sequencing, *Carassius auratus auratus*

Abstract

In the present study, the etiological agent that caused mortality in goldfish at an aquarium company located in Bursa in northwestern Turkey was identified. Diseased fish were lethargic, displaying a loss of appetite, ascites, and exophthalmia. Internal examination revealed that the kidney was surrounded by exudate and that the spleen and liver were pale. Microbiological examination showed that the kidney, spleen, and liver were infected by gram-negative, cytochrome oxidase, catalase, and H₂S positive rod-shaped bacteria identified as *Shewanella putrefaciens*. Identification of the bacteria was confirmed by 16S rRNA gene sequencing. Histopathological examination of the kidney, spleen, and liver revealed necrosis in kidney tubules and the presence of hepatocytes and hemosiderin in melanomacrophages. According to the disk diffusion method, isolates were sensitive to enrofloxacin (5 µg), florfenicol (30 µg), gentamycin (120 µg), and sulfamehoxazole+ trimethoprim (25 µg).

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Introduction

The genus *Shewanella* is affiliated with the subclass Protobacteria within the family Vibrionaceae (Ziemke et al., 1998). The first description of a species eventually assigned to this genus was provided in 1931 by Derby and Hammer who isolated an unknown bacterial taxon from putrefied butter and water supplies of dairies and named it *Achromobacter putrefaciens*; in 1941, Long and Hammer proposed that this species be transferred to the genus *Pseudomonas* under the name *P. putrefaciens* (Holt et al., 2005). In 1985, phylogenetic studies resulted in the reclassification of this organism to the family Vibrionaceae, and the description of a new genus, *Shewanella* (MacDonell and Colwell, 1985).

The genus *Shewanella* comprises gram-negative, oxidative, and non-oxidative bacilli widely distributed in marine and freshwater environments. *Shewanella putrefaciens* is generally isolated from sea water samples and putrefied fish (Shewan, 1977; Jergensen and Huss, 1989; Stenstrom and Molin, 1990). Beside aquatic reservoirs (sea water, fresh water, sewage), other isolation sources include natural energy reservoirs (oil and gas), poultry, meat, and milk products (Levin, 1972; Semple et al., 1989; Stenstrom and Molin, 1990; Vogel et al., 1997). Characteristic phenotypic attributes are the production of hydrogen sulfide gas on triple sugar iron (TSI) slants (Weyant et al., 1995; Ziemke et al., 1998) and good growth on conventional solid media, including MacConkey agar where the bacteria produce 1-2 mm yellowish-brown colonies after incubation for 18-24 h (Holt et al., 2005). In addition, *S. putrefaciens* produces acid from maltose and glucose and sometimes from sucrose and arabinose (Holt et al., 2005). Bacteria of the genus *Shewanella* are important in the turnover of organic material and capable of dissimilatory reduction of various metals and other substances such as nitrate, nitrite, thiosulphate, and trimethylamine-N-oxide (Holt et al., 2005).

The first isolation of *S. putrefaciens* from fish, more specifically from cultured rabbit fish (*Siganus rivulatus*), was achieved in 1987 by Saeed et al. (1990). Since then, the bacterium was isolated from common carp (*Cyprinus carpio* L.) and rainbow trout (*Oncorhynchus mykiss*) in Poland (Kozinska and Pekala, 2004) and from European sea bass (*Dicentrarchus labrax*) in Turkey (Korun et al., 2009).

The aim of the present study was to identify the causative agent of infection in five adult goldfish (*Carassius auratus auratus*) that were referred to the Aquatic Animal Diseases Department in Bursa, Turkey, after displaying a swollen abdomen, bilateral exophthalmia, abnormal swimming, loss of appetite, and stagnancy. Conventional tests, API 20 E rapid diagnostic kits, and VITEK II test kits were used to identify the cause of infection. The isolation of *S. putrefaciens* from goldfish shows the zoonotic ability of the bacterium.

Materials and Methods

Sampling and identification. In March 2012, five diseased and moribund adult goldfish (10-15 g) were transferred alive to our laboratory from an aquarium located in Bursa in northwestern Turkey and euthanized by an overdose of Quinaldine. Liver, spleen, and kidney tissue samples were streaked on tryptic soy agar (TSA), blood agar, and MacConkey agar and incubated 22°C for 48 h for bacteriologic isolation. The predominant colonies that grew on the plates were purified and identified by classic bacterial tests (Gram staining, motility, oxidation/fermentation, oxidase, catalase test, etc.). In addition, a commercial miniaturized API 20 E rapid test kit (bioMérieux, France) was used according to the manufacturer's instructions but with a modified incubation temperature (26°C for 24 h) and checked in APIweb, and VITEK II (bioMérieux, France) was used with a Gr(-) id card for 24 h incubation at 37°C (Whitman, 2004; Austin and Austin, 2007).

The identity of the isolated bacterium was confirmed by 16S rRNA gene sequencing (Suau et al., 1999; Zhang et al., 2000; Hall, 2004; CLSI, 2010). Proliferation in trypticase soy broth (TSB) with 0, 3%, 5%, 7%, or 9% NaCl and on thiosulfate citrate bile sucrose (TCBS) agar as well as hemolysis on sheep blood agar and sensitivity to 10 mg vibriostatic agent results were checked. FeS production on TSI agar medium (Difco)

was determined after incubation at 22°C for 48 h. The isolates were also incubated on TSA medium for 5 days to check their ability to proliferate at 5°C, 22°C, 37°C, and 42°C.

Genomic DNA extraction. The isolated strain was inoculated into nutrient broth and incubated overnight at 22°C in a shaking incubator. Genomic DNA was extracted with a High Pure PCR Template Preparation Kit (Roche Applied Science) according to the manufacturer's directions and used as the template for PCR.

PCR and 16S rRNA gene sequencing. Genomic target DNA was amplified using the 16S rRNA gene universal bacteria-specific primer set: primer S-20 - 5' AGA GTT TGA TCC TGG CTC AG 3' and primer A-18 - 5' GWA TTA CCG CGG CKG CTG 3' (Suau et al., 1999). The PCR mixture (50 µl) included approximately 100 ng template DNA (2 µl), 10 pmol of each primer (2 x 2 µl), 2X PCR Master Mix (Fermentas; 25 µl), and nuclease-free water (Fermentas; 19 µl). Amplification was performed using a thermal cycler (Biometra) with the following parameters: initial denaturation at 95°C for 3 min, followed by 30 cycles of amplification (denaturation at 95°C for 30 s, primer annealing at 56°C for 1 min, extension at 72°C for 1 min), and a final extension step of 72°C for 4 min. After amplification, 10 µl of the PCR sample was loaded on a 1.5% (wt/vol) agarose gel in Tris-acetate-EDTA containing ethidium bromide (0.5 µg/ml) and electrophoresis was performed for 40 min at 90 V. PCR products were visualized on a UV transilluminator and size was estimated against the GeneRuler 100 bp DNA Ladder (Fermentas SM0241). PCR products were cleaned and sequenced by BM Labosis (Ankara, Turkey) using the given primers. Sequence editing and analysis was performed using Bioedit v7.0.0 (Hall, 2004) and BLASTN 2.2.20 algorithm (Zhang et al., 2000).

Histopathological examination. Spleen, kidney, and liver tissue samples were taken during necropsy. The samples were fixed in a 10% buffered formalin solution for 24 h and further processed following standard techniques. The tissue samples were embedded in paraffin wax, cut into 5-µm thick sections, mounted on slides, stained with hematoxylin-eosin (H&E) and Brown & Brenn stains for bacteria, and examined under a light microscope.

Determination of antimicrobial sensitivity. The bacteria isolates were tested for antimicrobial susceptibility by the disc diffusion method on Mueller-Hinton agar and incubated at 22°C for 24 h. The tested antibiotics (Oxoid) included amoxicillin (25 µg), doxycycline (30 µg), enrofloxacin (5 µg), erythromycin (15 µg), florfenicol (30 µg), gentamycin (120 µg), lincomycin (2 µg), neomycin (10 µg), oxytetracycline (30 µg), and sulfamethoxazole+trimethoprim (25 µg). Incubation zone diameters were measured after 24 and 48 h of incubation and classified as sensitive (S) or resistant (R) according to the size of the zone of bacteria growth inhibition according to the Clinical and Laboratory Standards Institute (CLSI, 2010). In addition, ATB VET (bioMérieux) test was carried out to determine sensitivity of the bacteria to different antibiotic agents.

Results

Clinical and autopsy findings. The diseased fish displayed stagnancy, abnormal swimming, lack of appetite, swelling of the abdomen, and bilateral exophthalmia (Fig. 1). Internal examination revealed a pale liver as well as ascites and enlargement of the spleen. The kidney was difficult to identify due to exudate. Pure culture colonies were opaque, 1-2 mm in diameter, smooth, slightly convex, and had a pinkish color with a bright surface on TSA plates after 48 h at 22°C. Bacteria appeared as gram-negative rods after Gram staining, were cytochrome oxidase and catalase positive, and were motile according to the hanging drop motility test (Table 1). The oxidation-fermentation test revealed that the bacteria were nonoxidative. No hemolysis was seen on sheep blood agar. The isolate was resistant to 10 mg/ml vibriostatic agent and grew in TSB with 0%, 3%, 5%, or 7% NaCl but not 9% NaCl or on TCBS agar.

API 20E rapid test and VITEK II GR(-) id card. In addition to conventional tests, API 20 E and VITEK II tests identified the isolate as *S. putrefaciens* with 98.3% and 94% probability, respectively (Table 1).



Fig. 1. Asymmetry and ascites in goldfish caused by *Shewanella putrefaciens*.

Gene sequencing. Our partial 16S rRNA gene sequence was identical to *S. putrefaciens* strain HAC411 (DQ307731) and showed 99% similarity to *S. putrefaciens* strain N22 (AB182185), isolated from the intestinal tract of carp, *Cyprinus carpio* (Namba et al., 2007). The isolated strain was named UAAD7 and the corresponding partial 16S rRNA gene sequence was deposited in the GenBank database under accession number JX518487.

Histopathological findings. Histological examination of the goldfish naturally infected with *S. putrefaciens* indicated depletion of white and red pulp and deposition of hemosiderin in melanomacrophage centers in the spleen (Fig. 2). The kidney was severely affected, displaying necrosis in renal tubules (Fig. 3). In addition, the kidney was swollen and

Table 1. Phenotypic characterization and API 20E test results of *Shewanella putrefaciens* isolated from goldfish and compared with earlier studies.

Phenotypic character	Our <i>Shewanella putrefaciens</i>	Korun et al. (2009) isolate	Saeed et al. (1987) isolate	Kozinska and Pekala (2004) isolate
Gram staining	-	-	-	-
Cytochrome oxidase	+	+	+	99**
Catalase	+	+	nd	nd
Oxidation/fermentation	-/-	-/-	-/-	-/-
Mobility	+	+	+	+
Growth on MacConkey agar	+	nd	nd	+
Hemolysis on blood agar	-	+	nd	nd
Growth on TCBS agar	-	+	nd	nd
O/129 (10 µg/disc)	-	+	nd	nd
<i>Growth on TSB agar in...</i>				
0% NaCl	+	+	-	+
3% NaCl	+	+	nd	nd
5% NaCl	+	+	+	+
7% NaCl	+	+	+	60**
9% NaCl	-	nd	+	60(w)**
5°C	+	+	nd	+
<i>Growth on TSA agar at...</i>				
22°C	+	+	nd	nd
37°C	+	+	+	+
42°C	-	nd	+	-
FeS production on TSI agar	+	nd	nd	nd
<i>API 20E test results</i>				
Beta galactosidase	-	-	-	-
Arginin dihidrolaz	-	-	-	-
Lysine decarboksilase	-	-	-	-
Ornithine decarboxylase	-	+	+	80**
Citrate utilization	+	+	+	75**
H ₂ S utilization	+	+	+	75**
Urease utilization	-	-	-	1**
Tryptophan deaminase	-	nd	-	-
Indole utilization	-	-	-	-
Voges Proskauer	-	-	-	-
Gelatin hydrolysis	-	+	+	75**
Glucose*	+	-	-	1**
Mannitol*	-	-	-	-
Inositol*	-	-	-	-
Sorbitol*	-	nd	-	-
Ramnose*	-	nd	-	-
Sucrose*	-	-	-	1**
Melibiose*	-	nd	-	-
Amygdalin*	-	nd	-	-
Arabinose*	-	-	-	2**
NO ₂	+	+	+	96**
N ₂	-	nd	nd	nd

* acid utilization from carbohydrates

** percent of positive isolates/strains

nd = no data

w = weak growth

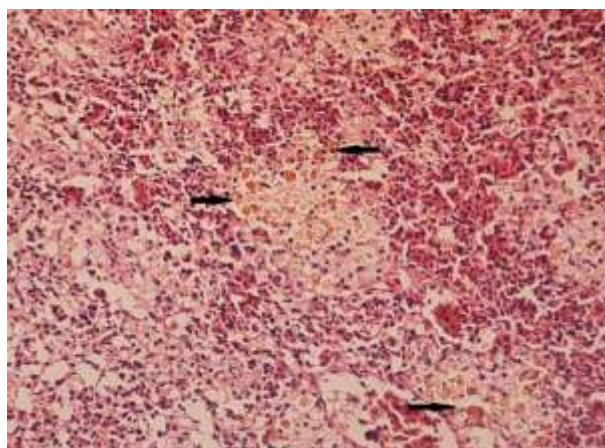


Fig. 2. Hem siderin in melanomacrophages in the center of the spleen (arrows) of goldfish infected by *Shewanella putrefaciens*, H&E stain.

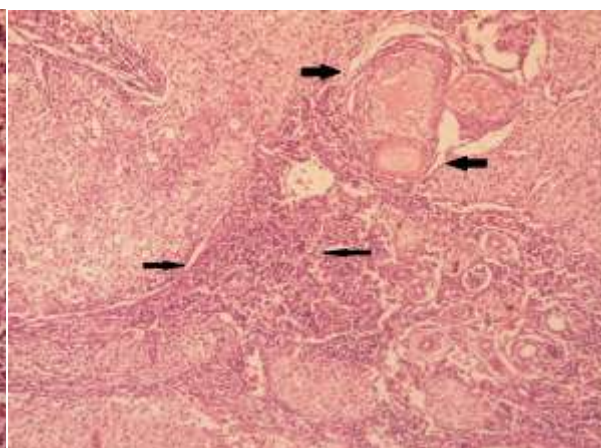


Fig. 3. Necrotic areas (thick arrows) and mononuclear cell infiltrations (thin arrows) in the kidney of goldfish infected by *Shewanella putrefaciens*, H&E stain.

there was exudate on the cut surface. There were mononuclear cells and connective tissue around the necrotic areas on the kidney (Fig. 4). Similar areas were observed on liver samples.

Antimicrobial sensitivity. According to the disk diffusion method, the isolated strain was resistant to amoxicillin, doxycycline, lincomycin, and neomycin, but sensitive to enrofloxacin, florfenicol, gentamycin, and sulfamethoxazole+trimethoprim. Results of the ATB VET test are given in Table 2.

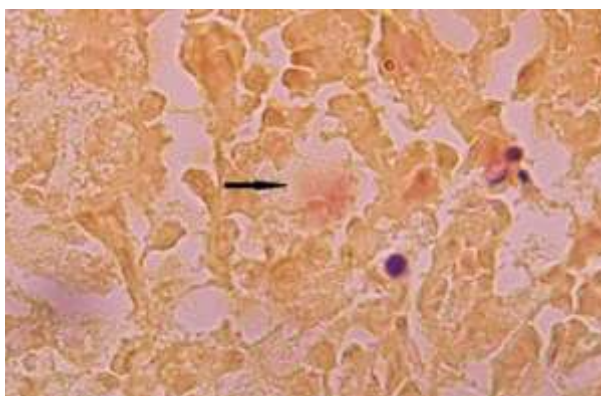


Fig. 4. *Shewanella putrefaciens* in the kidney tissue of goldfish, B&B stain.

Table 2. Antibiotic sensitivity results of *Shewanella putrefaciens* using ATB VET (bioMérieux, France).

Antimicrobial drug	mg/l	Result	Antimicrobial drug	(mg/l)	Result
Amoxicillin	4	R	Kanamycine	8	R
Amoxicillin/clavulanic acid	4/2	R	Lincomycine	2	R
Apramycine	16	S	Metronidazole	4	R
Cefoperazone	4	S	Nitrofurantoin	25	S
Cefalotine	8	R	Oxacilline	2	R
Chloramphenicol	8	S	Oxolinic acid	2	S
Colistine	4	S	Penicillin	0.25	R
Cotrimoxazole	2/38*	S	Pristinamycine	2	R
Doxycycline	4	R	Rifampicine	4	S
Enrofloxacin	0.5	S	Spectinomycine	64	R
Erythromycine	1	R	Streptomycine	8	R
Flumequine	4	S	Sulfamethizole	100	R
Fucidic acid	2	R	Tetracycline	4	R
Gentamycin	4	S	Tylosine	2	R

R = resistant, S = sensitive

* 2 mg trimethoprim and 38 mg sulfamethoxazole/l

Discussion

Shewanella putrefaciens is an opportunistic bacterial pathogen that can cause disease in fish under stressful conditions (Kozinska and Pekala, 2004). Human infections by members of the genus *Shewanella* are rare, and have been reported mainly from warm climatic areas in the USA, Australia, Asia, South Africa, and southern Europe or during especially warm summers in temperate climates such as that of Denmark (Holt et al., 2005). Immunosuppressive people with pre-existing cutaneous ulcers should be especially careful of Shewanellosis after exposure to sea water or a marine environment (Tsai et al., 2008). As *S. putrefaciens* has zoonotic potential, any direct contact between humans and infected aquatic animals such as the infected goldfish in the present study is

risky (Chen et al., 1997).

Shewanella algae has been misidentified as *S. putrefaciens* as a result of the failure of conventional phenotypic characteristic testing and commercial bacteria identification systems such as ID 32 GN and VITEK to differentiate *S. putrefaciens* from *S. algae* (Tsai et al., 2008). Therefore, we used sequencing to confirm the identity of our conventionally-isolated isolate. Our isolate was correctly identified by test systems and our partial 16S rRNA gene sequence was identical to an *S. putrefaciens* entry in the EMBL-bank but had only 91.3% sequence similarity (excluding the missing bp's) to the *S. algae* type strain LMG 18393 (accession no. AF005249).

While our *S. putrefaciens* isolate showed homogeneity with earlier studies (Saeed et al., 1990; Kozinska and Pekala, 2004; Korun et al., 2009), API 20E and conventional tests found differences in hemolysis on blood agar, growth on TCBS agar, O/129 (10 µg/disc), 9% NaCl, 42°C, ornithine decarboxylase, gelatin hydrolysis, and acid production from glucose, indicating that there may be variation between *S. putrefaciens* strains regarding decarboxylase, gelatin, and glucose metabolism. The *S. putrefaciens* isolate isolated from freshwater fish by Kozinska and Pekala (2004) showed negative hemolysis, good growth in TSB without NaCl, and weak growth in TSB with 9% NaCl, similar to our results. However, *S. putrefaciens* isolated from diseased rabbit fish did not grow in TSB with 1% NaCl but did grow in TSB with 9% NaCl (Saeed et al., 1990). Taken together, these differences suggest that some strains may have adapted to fresh water. Our isolated bacterium grew at 5°C, 22°C, and 37°C but not at 42°C, consistent with the findings of Kozinska and Pekala (2004) but different from those of Saeed et al. (1990), indicating that *S. putrefaciens* isolated from freshwater fish and those isolated from marine fish may have different phenotypes.

Inappetence, skin ulcers, darkening of the skin, exophthalmia, and ascites were reported in outbreaks of *S. putrefaciens* (Saeed et al., 1990; Kozinska and Pekala, 2004; Korun et al., 2009). In our study, hemosiderin was deposited in melanomacrophages on red and white pulpas of the spleen and there were mononuclear cells around necrosis on liver and renal tubules of diseased goldfish. Our findings agree with other histopathological examinations related to infection with this bacterium in different fish (Saeed et al., 1990; Kozinska and Pekala, 2004; Korun et al., 2009). Our *S. putrefaciens* isolate was sensitive to enrofloxacin, florfenicol, oxolinic acid, and oxytetracycline, frequently used antibiotics in aquaculture. These results are very similar to antibiogram findings of Kozinska and Pekala (2004) - sensitivity to enrofloxacin, flumequine, gentamycin, and oxytetracycline. However, while our isolate is resistant to erythromycin and kanamycin, the isolate of Korun et al. (2009) is sensitive to these antibiotics.

In conclusion, the histopathological changes and antibiotic susceptibility of our *S. putrefaciens* isolate are in accordance with previous reports but results of some biochemical tests indicate a variation between *S. putrefaciens* strains. The identification of *S. putrefaciens* in ornamental fish is important due to the bacterium's zoonotic potential. Thus, the pathogenesis of *S. putrefaciens* and factors affecting the transmission of Shewanellosis infection should be of interest and further studied.

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