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Diagnosis of Vibriosis in Humpback Red Snapper (*Lutjanus gibbus* Foorsskal, 1775) and Golden Trevally (*Gnathanodon speciosus* Foorsskal, 1775) in a Marine Aquarium, Turkey

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ABSTRACT

This study was carried out to determine the reason of mortalities in humpback red snapper (*Lutjanus gibbus*) and golden trevally (*Gnathanodon speciosus*) kept in a marine aquarium in Turkey. Moribund fish showed diffuse haemorrhages on upper and lower jaws, superficial and ulcerative skin lesions, tail rot and pale gills. Internally, clear fluid in peritoneal cavity and pale liver were observed. The isolated bacteria were identified as *Vibrio harveyi* according to their morphological, physiological, biochemical characteristics and API 20E results. These bacterial identifications were also confirmed with 16S rRNA gene sequence analysis. The isolates were determined to be sensitive to sulphamethoxazole/ trimethoprim, florphenicol and flumequine and were resistant to erythromycin, kanamycin and ampicillin. *Vibrio harveyi* infected fish generally showed epithelial hyperplasia in the gill filaments, ulcerative skin lesions, degeneration or necrosis of the hepatic cells, kidney tubules and parenchyma cells of the spleen, multifocal melanomacrophage centres in the kidney and reduced hemopoietic tissue in the spleen.

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Türkiye'de Bir Deniz Akvaryumunda Humpback Red Snapper (*Lutjanus gibbus* Foorsskal, 1775) ve Golden Trevally'de (*Gnathanodon speciosus* Foorsskal, 1775) Vibriozisin Teşhisi

ÖZET

Bu çalışma, Türkiye'de bir deniz akvaryumunda bulunan humpback red snapper (*Lutjanus gibbus*) ve golden trevally (*Gnathanodon speciosus*) balıklarının ölüm nedenlerinin belirlenmesi amacıyla yapılmıştır. Hasta balıklarda, üst ve alt çenelerde yaygın hemoraji, yüzeysel ve ülseratif deri lezyonları, kuyruk yüzgecinde erime ve solungaçların solgun olduğu gözlemlendi. İç bakıda, periton boşluğunda berrak sıvı birikmesi ve solgun karaciğer tespit edildi. İzole edilen bakteriler morfolojik, fizyolojik, biyokimyasal özelliklerine ve API 20E sonuçlarına göre *Vibrio harveyi* olarak tanımlandı. Bu bakteriyel tanımlamalar 16S rRNA gen dizisi analizi ile de doğrulanmıştır. İzolatların sülfametoksazol/trimetoprim, florfenikol ve flumequin'e duyarlı oldukları ve eritromisin, kanamisin ve ampisiline dirençli oldukları belirlendi. *Vibrio harveyi* ile enfekte olmuş balıklarda genellikle solungaç filamentlerinde epitel hiperplazisi, ülseratif deri lezyonları, hepatik hücrelerde, böbrek tübüllerinde ve dalağın parankim hücrelerinde dejenerasyon veya nekroz, böbrekte multifokal melanomakrofaj merkezleri ve dalakta hemopoietik dokuda boşalma tespit edildi.

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INTRODUCTION

The humpback red snapper (*Lutjanus gibbus*) and the golden trevally (*Gnathanodon speciosus*) are reef-dwelling marine species occurring in the tropical and subtropical waters of the Eastern Pacific, Western Indo-Pacific and Eastern and Western Atlantic Ocean. They are farmed in cage aquaculture by Singapore, Taiwan, Malaysia and Indonesia and both of them are important marine species in the aquarium industry (FAO, 2016).

Vibriosis is one of the most prevalent fish diseases caused by bacteria belongs to the genus *Vibrio* and the genus includes more than hundred species and diseases caused by the vibrios was reported in different marine, freshwater and aquarium fish species. *Vibrio harveyi* is a marine Gram-negative luminous organism with a requirement for sodium chloride belong the genus *Vibrio* (Cano-Gomez *et al.*, 2009). *Vibrio harveyi* was reported in various Sparidae family members (Balebona et al., 1998, Pujalte et al., 2003, Haldar et al., 2010) as well as cultured common dentex (*Dentex dentex*) (Company et al., 1999, Pujalte et al., 2003, Turgay and Karataş, 2016), sole (*Solea senegalensis* and *Dicologoglossa cuneata*) (Zorilla et al., 2003, Lopez et al., 2009) and chub mackerel (*Scomber japonicus*) (Lee et al., 2016). It has also become recognized as a serious cause of diseases invertebrates and especially caused important economic losses of penaeid shrimp (Austin and Zhang, 2006). The most common disease symptoms are haemorrhages and superficial skin lesions, and in most cases, a general septicaemia (Cano-Gomez *et al.*, 2009).

In Turkey, *V. harveyi* was firstly reported blue carp (*Callinectes sapidus*) in Antalya (Yalçınkaya *et al.*, 2003). Later than, the bacterium was also reported as a secondary pathogen in diseased European sea bass (*Dicentrarchus labrax*) and associated with infestation by *Ceratothoa oestroides* (Korun and Akaylı, 2004) and later aetiological agent of vibriosis with other *Vibrio* species in diseased European sea bass in Turkey by Korun and Timur (2008). Another outbreak caused by *V. harveyi* was described with haemorrhage, scale rots on the skin surface, depigmentation, pale gills, corneal opacity and swollen intestine as common findings in common dentex (*Dentex dentex*) by Turgay and Karataş (2016).

The recirculation aquaculture systems (RAS) especially marine aquarium water, contain substantial number of bacteria. Raja et al. (2006) declared that the presence of high concentration of *Vibrio* species in the recirculated marine aquarium systems. They were also related with water quality and quantity. While the bacteria were introduced into the systems from uneaten feed or diets, dead or wounded fish, feces etc., increasing of bacterial population might be affected fish heath. When aquarium fish placed in aquaria, many disease problems may be seen by arising artificial conditions. In this paper we described vibrosis causing mortality due to *Vibrio harveyi* infection in humpback red snapper and golden trevally from a marine aquarium in Turkey.

MATERIAL METHODS

The Water Parameters and Sampling

The water parameters of the public aquarium were measured as: water temperature 24˚C, salinity 28‰, pH 8, dissolved oxygen concentration 7,6 mg/l and oxidation/reduction potential 175mV. Two moribund fish were sampled for each species from the same public aquarium located in Istanbul, Turkey. Bacteriological samples taken from visceral organs and blood were streaked onto Marine Agar (MA) and Thiosulfate Citrate Bile Salts Sucrose Agar (TCBS) plates and the plates were incubated at 22˚C for 48h. The morphological and physiological characteristics of the bacteria were determined by using conventional biochemical and physiological test and rapid identification kits (API 20E) according to Buller (2004) and Austin and Austin (2007). For histopathological examination, tissue samples were fixed in 10% buffered formalin and processed for paraffin embedding. The histological sections (5µm) were stained with Haematoxylin and Eosin and examined under a microscope (Bullock, 1978).

DNA Isolation from Bacteria and 16S rRNA Gene Sequencing

Pure bacterial cultures were selected and the isolates were inoculated into Marine Broth 2216 and incubated overnight at 22°C. Total DNA extraction was performed with the GeneJET Genomic DNA Purification Kit according to the manufacturer's instructions and used as template for PCR. A partial 16S rRNA gene was amplified using the universal bacteria 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) were prepared using PCR Master Mix (2X) according to manufacturer's instructions. The amplification was done using a thermal cycler and a program with the following parameters: initial denaturation at 95°C for 3 min, followed by 25 cycles of amplification (denaturation at 95°C for 30 s, 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. PCR products were sequenced bidirectionally by Medsantek (Istanbul, Turkey). Sequence editing and analysis was performed in Bioedit v7.0.0 (Hall, 1999) using the ClustalX 2.1 (Larkin et al., 2007) and BLASTN 2.2.20 algorithm (Zhang et al., 2000).

Antimicrobial Susceptibility Test

All isolates were tested for antimicrobial susceptibility by the disc diffusion method on Marine Agar. The

antimicrobial agents tested were as follows: flumequine (30 µg), kanamycin (30 µg), oxytetracycline (30 µg), florphenicol (30 µg), chloromphenicol (30 µg), erythromycin (15 µg), sulfamethoxazole/trimethoprim (1.25 µg/ 23.75 µg), ciprofloxacine (1 µg), enrofloxacine (5 µg) and ampicillin (10 µg). The isolates were classified as sensitive (S), intermediary sensitive (I), or resistant (R) on the basis of the size of the zone of bacterial growth inhibition, according to the Clinical and Laboratory Standards Institute (CLSI, 2010).

RESULTS and DISCUSSION

Moribund fish showed anorexia, diffuse haemorrhages on upper and lower jaws (Figure 1A, 1B), depigmented area on skin (Figure 1B), superficial and ulcerative skin lesions (Figure 1C), pale gills (Figure 1D), frayed fins (Figure 1D, 1E), tail rot (Figure 1E), erythema in dorsal fins (Figure 1E) and enlarged abdomen. Internally clear fluid in peritoneal cavity and pale liver were observed (Figure 1F). After 48h incubation at 20°C, yellowish coloured colonies were growth on TCBS plates. All isolates were determined as Gram negative, motile, cytochrome oxidase and catalase positive, fermentative and sensitive to O/129. The basic characteristics are shown in Table 1. According to their morphological and biochemical characteristics and 16S rRNA gene sequencing results, all isolated bacteria were identified as *Vibrio harveyi* (Accession numbers: KY393105-KY393108, KY974264-KY974266).

In our study, we isolated nine *Vibrio harveyi* strains from diseased fish. In our study, all isolates showed straight edge, yellowish colonies on TCBS plates as described in other studies (Austin and Austin, 2004, Buller, 2004, Austin, 2010, Turgay and Karataş 2016). Although degradation of urea, Voges Proskauer reaction and ß-galactosidase (ONPG) were found variable, all *V. harveyi* isolates utilized cellobiose as a carbon source and were able to decarboxylate ornithine and lysine. Similar to this, in another study (Liu et al. 2004), degradation of urea and ß-galactosidase (ONPG) were found variable in *V. harveyi* strains isolated from diseased cobia.

Table 1. Morphological and phenotypical characteristics of *Vibrio harveyi* isolates

Fish	Golden trevally					Humpback red snapper			
Isolate Number	1	2	3	4	5	6	7	8	9
Isolated Organs	spleen	spleen	blood	kidney	liver	blood	liver	blood	spleen
Morphology	B	B	B	B	B	B	B	B	B
Motility	+	+	+	+	+	+	+	+	+
Gram Staining	-	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+	+
Cytochrome Oxidase	+	+	+	+	+	+	+	+	+
O/129 Resistance (150ig)	S	S	S	S	S	S	S	S	S
Growth on TCBS	Y	Y	Y	Y	Y	Y	Y	Y	Y
O/F	F	F	F	F	F	F	F	F	F
Indole	+	+	+	+	+	+	+	+	+
Voges Proskauer Reaction	-	-	-	-	-	-	+	+	+
Methyl Red	+	+	+	+	+	+	+	+	+
Nitrate Reduction	+	+	+	+	+	+	+	+	+
Arginine dihydrolase	-	-	-	-	-	-	-	-	-
Lysine Decarboxylase	+	+	+	+	+	+	+	+	+
Ornithine Decarboxylase	+	+	+	+	+	+	+	+	+
Gelatine	+	+	+	+	+	+	+	+	+
Citrate	+	+	+	+	+	+	+	+	+
Degradation of Urea	-	-	-	-	-	-	+	-	-
ONPG	-	-	-	-	-	-	+	+	+
Esculine dihydrolyse	+	+	+	+	+	+	+	+	+
Acid Production of									
Glucose	+	+	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	+	+
Arabinose	+	+	+	+	+	+	+	+	+

B: bacillus; +: positive, -: negative; S: sensitive; Y: yellow; F: fermentative

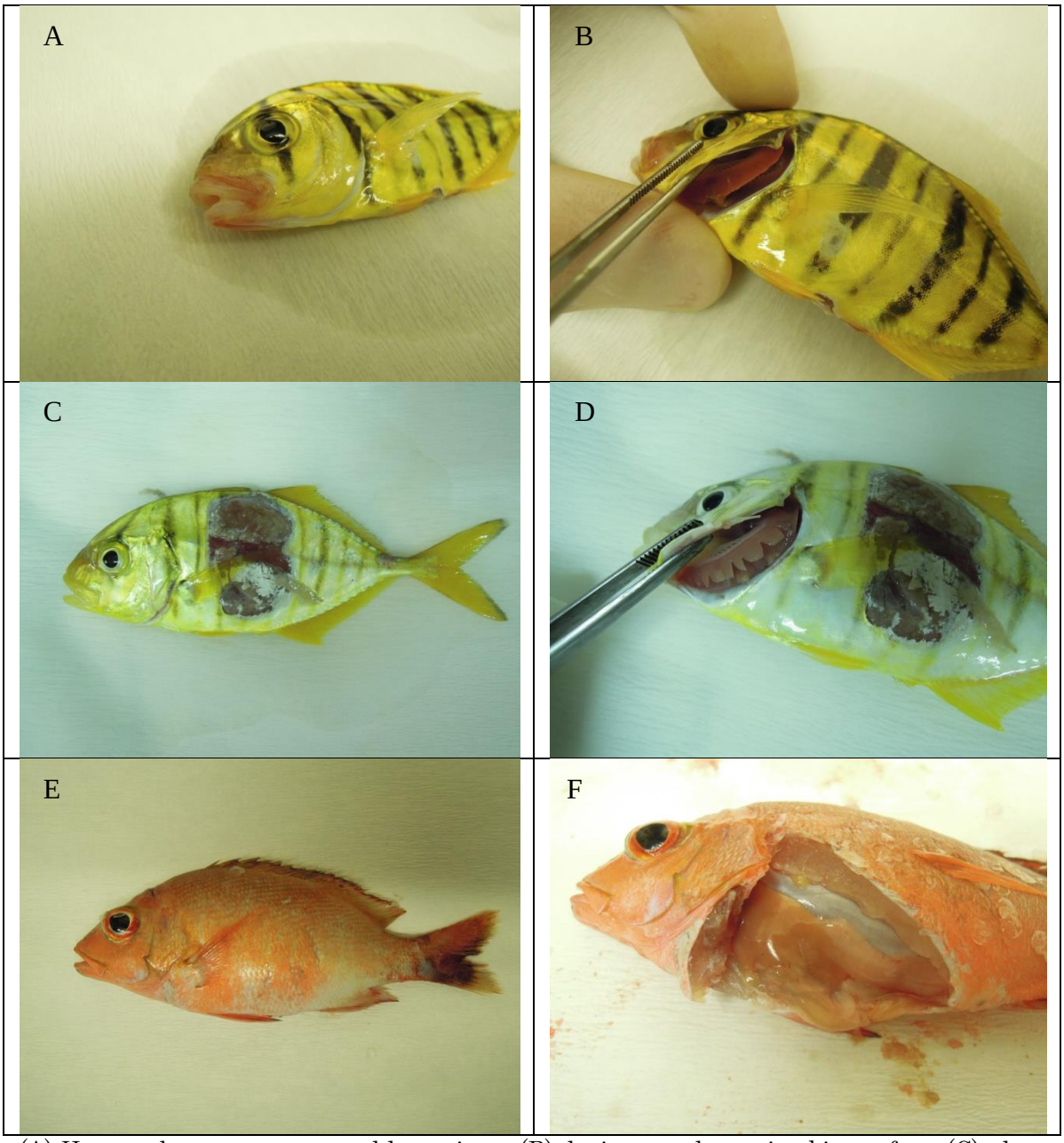

Figure 1. (A) Haemorrhages on upper and lower jaws, (B) depigmented area in skin surface, (C) ulcerative skin lesions, (D) pale gills, (E) erythema in frayed fins and tail rot, and (F) pale liver.

Vibrio species cause infections in fish under stress in culture conditions and clinical signs may vary depending on the form of the infection (per-acute, acute or chronic). The most characteristic clinical sign of vibriosis includes haemorrhages, skin lesions, releasing blood exudates, corneal lesion such as general septicaemia. However, in acute form, mortality can be seen without showing any clinical sings (Karataş and Candan, 2007). Diffuse haemorrhages on upper and lower jaws, skin lesions, pale gills, frayed fins, tail rot and enlarged abdomen such as clear fluid in peritoneal cavity and pale liver were clinically observed in the moribund fish. These findings were found so similar with acute form of vibriosis as described in some other studies (Lee at al., 2016, Turgay and Karataş, 2016). Liu *et al.* (2004) also determined ascites and gastroenteritis exhibited as distended abdomen and anus in the moribund cobia infected with *V. harveyi*. We reported similar ascites in affected fish.

Histopathologically, the affected fish showed ulcerative skin lesions, degeneration or necrosis of the hepatic cells (Figure 2A), the kidney tubules (Figure 2B) and the parenchyma cells of spleen (Figure 2C). There were multifocal melanomacrophage centres in the kidney (Figure 2B) and reduced haemopoietic tissue in the spleen.

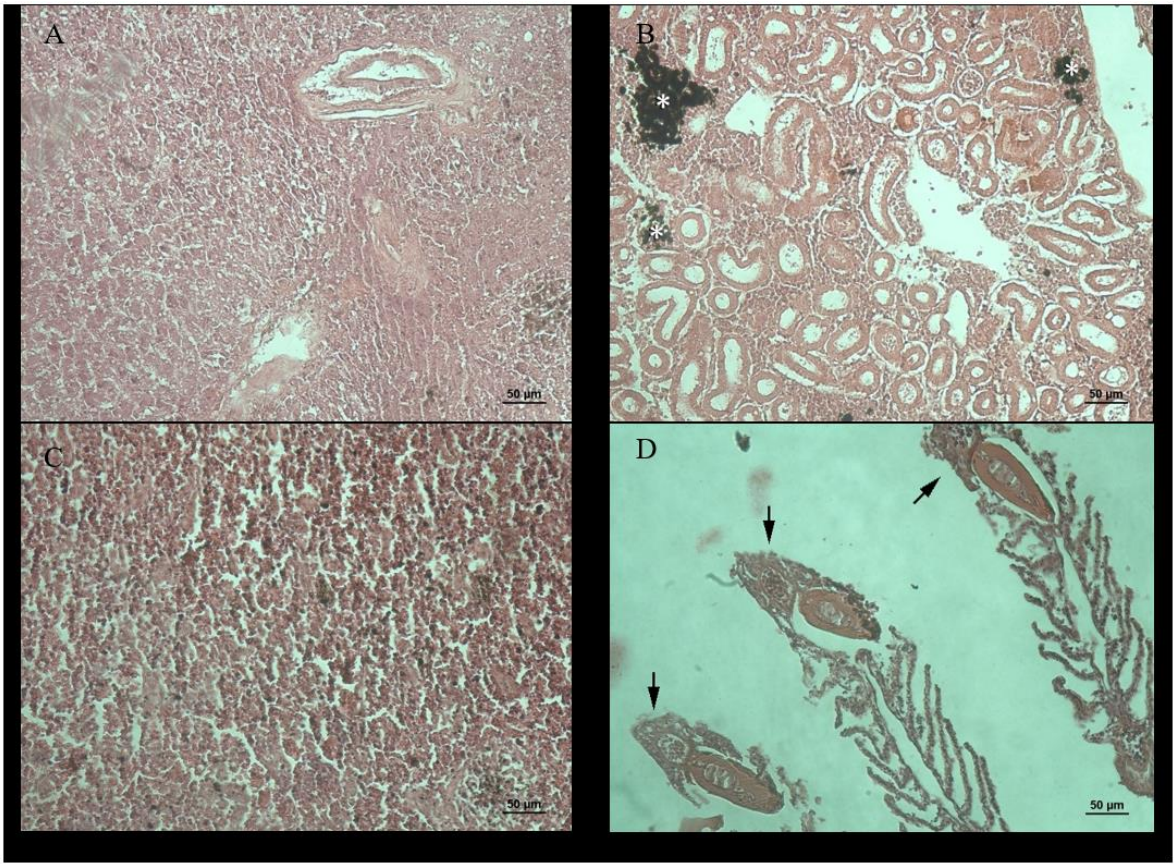

Figure 2. (A) Degeneration or necrosis of hepatic cells, (B) multifocal melonamacrophage centres (asterisk) and necrotic tubules in kidney, (C) reduced haemopoietic tissue in the spleen, (D) epithelial hyperplasia and haemorrhages of the necrotic gill filaments tips (arrowed).

Epithelial hyperplasia, haemorrhages of the necrotic gill filaments tips (Figure 2D) were also observed in gill filaments of each affected fish species. Korun and Timur (2008) also reported similar histopathological changes in European sea bass infected with *V. harveyi* and other *Vibrio* species. Similar histopathological changes were observed in this study.

A study relating the susceptibility of *V. harveyi* strains to antibiotics by Liu et al. (1997) showed that three strains isolated in Taiwan were found resistant against nitrofurantoin, novobiocin and sulphonamide. In another study (Liu et al., 2004), ten *V. harveyi* isolates were examined and all strains were found resistant against ampicillin, penicillin and vancomycin. Ampicillin, flumequine, furazolidone, oxolinic acid and sulfamethoxazole/trimethoprim were found the most effective chemicals to vibriosis treatment by Korun and Timur (2008). In another study, Lee et al. (2016) determined that *V. harveyi* strains were resistant to fluoroquinolones. In this study, we determined that all strains were found resistant against erythromycin, kanamycin and ampicillin and the isolates were sensitive and intermediary sensitive to flumequine, oxytetracycline, florphenicol, chloromphenicol, sulfamethoxazole/ trimethoprim, ciprofloxacine, enrofloxacine, therefore they can be suggested to use in disease control.

CONCLUSION

In conclusion, acute vibrosis causing mortality due to *Vibrio harveyi* infection in humpback red snapper and golden trevally was diagnosed in a marine aquarium in Turkey.

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