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Vibriosis as an Epizootic Disease of Rainbow Trout (*Onchorhynchus mykiss*) in Turkey

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Abstract: *Vibrio anguillarum* O1 was isolated from diseased cultured rainbow trout *Onchorhynchus mykiss*, in the Southern Aegean of Turkey with lesions and signs of vibriosis. Gram (-), motile and curved rods from diseased rainbow trout were identified after physiological, biochemical and slide agglutination tests as *V. anguillarum* O1. Koch postulates were fulfilled with the isolated bacteria. The antibiotic susceptible test results showed that *V. anguillarum* strains were susceptible to amoxicillin, ampicillin, flumequin, furazolidone, kanamycin, potentiated sulphonamide and oxolinic acid.

Key words: *Vibrio anguillarum*, *Onchorhynchus mykiss*, vibriosis

INTRODUCTION

Vibriosis is one of the most prevalent fish diseases caused by bacteria belonging to the genus *Vibrio*. Vibriosis caused by *Vibrio anguillarum* has been particularly devastating in the marine culture of salmonid fish. The causative agent, *V. anguillarum*, was first described in 1909 by Bergman as the aetiological agent of the red pest of eels in the Baltic Sea. Vibriosis was not reported in North America until 1953.

When *V. anguillarum* was isolated from chum salmon (*Oncorhynchus keta*). Outbreaks affecting close to 50 species of fresh and salt water fishes have been reported in several countries in the Pacific, as well as the Atlantic coasts (Anderson and Conroy, 1970; Strout *et al.*, 1978; Winton *et al.*, 1983; Tolmasky *et al.*, 1985). In Japan, vibriosis affects freshwater fish, especially ayu caused by *V. anguillarum* serotype J-O-1 in Lake Biwa. In July 1990 the same disease killed more than 1.6 million feral ayu *V. anguillarum* has been demonstrated that the two serogroups, while O3 has been frequently isolated from diseased eels (*Anguilla anguilla*) (Larsen *et al.*, 1994).

V. anguillarum was serotyped from O1 to O16 by Grisez and Ollevier (1995). Later, *V. anguillarum* strains, which were isolated from various fish species in all over the world, were reported in 23 *O. serogroups* (Pedersen *et al.*, 1999).

V. anguillarum, also cause disease in marine fish aquaculture in Turkey (Cagirgan, 1993; Tanrikul *et al.*, 2005) has been known for along time, but has not been previously reported from rainbow trout (*Oncorhynchus mykiss*) in freshwater farming now causes many problems. And the infection spreads rapidly in rainbow trout farms.

The objective of this study was to determine, diagnosis confirmed serological and biochemical characteristics of *V. anguillarum* species. In addition, antibiogram results of isolated bacteria from fish farms are given. The strains included in this study were also selected based on diversity of geographical origin and in biochemical reactions that resulted from the preliminary identification.

MATERIALS AND METHODS

Fish: Fish samples were made on juvenile and adult rainbow trout (*Onchorhynchus mykiss*), ranging from 20 to 300 g of body weight from 8 different fish farm in Southern Aegean Region of Turkey. Recently died or moribund fish from 12 disease cases involving rainbow trout, were brought to Ege University, Fisheries Faculty, Department of Fish Disease's Laboratory for disease diagnosis. Samples came from 8 fish farms, 6 land-based and 2 cage farms, during a 3 year period (2001-2004). The postmortem examinations of external and internal disease signs were performed.

Bacterial strains:

Isolation and selection: Kidney samples of 10 fish were inoculated onto Tryptic Soy Agar (TSA) (Oxoid) and Brain-Heart Infusion agar (BHA) (Oxoid), incubated at 21°C and examined daily for 3 days. Representative colonies on TCBS (Oxoid) were selected for purity. Those presumptively diagnosed as *Vibrio* sp. selected for further identification.

Preservation: Stock cultures were maintained at 4°C or at room temperature on TSA (Oxoid) and reinoculated every 1 or 2 months. All long-term storage of bacterial

strains, were kept at -30°C on tryptic soy broth (Merc) supplemented with 10% glycerol.

Biochemical and physiological tests presumptive identification: Colony morphology was observed on TSA (Oxoid) and BHA (Oxoid) after 2 days of incubation at 21°C. Gram staining and oxidase test were carried out according to standard procedures. Motility was determined by the hanging drop method. Oxidative and fermentative degradation of glucose and gas production were tested on MOF medium (Difco). Sensitivity to the vibriostatic agent was tested with discs (Oxoid) on TSA (Oxoid).

Further identification: Young cultures were used for the biochemical tests and incubated at 21°C. TSA was used as a base in all test media except for growth tests at different salt concentration. The salt requirement was tested using peptone water. Biochemical tests carried out in API 20E systems (BioMerieux S.A). The incubation was 72 h at 21°C.

Challenge tests : PBS was used for all suspensions and dilutions of bacteria in the challenge tests and for the viable counts. A bacterial suspension in 1 mL PBS was inoculated onto two TSA with a pasteur pipette, excess liquid removed and the plates dried. The growth was harvested with 3 mL of PBS after 19 h incubation at 21°C. Serial 10 fold dilutions were prepared and kept on ice and the fish challenged with 1 h.

Rainbow trout parr of ≈50 g from a smolt-producing facility with no known history of disease were transferred to the experimental facility 7 days before challenge. The fish were kept in freshwater 15°C.

The experiments using 1 no isolate were terminated and an attempt to determine LD50 was made using 1 No. isolate only. Thirty fish were inoculated with dilution of bacteria and 30 fish with PBS for control. The fish were injected intraperitoneally with 0.1 mL, such that the dose per fish was 1.6×10^6 CFU. The fish were observed daily during the experiment, dead samples were taken aseptically from.

Preparation of antisera and slide agglutination test: Slide agglutination test were carried out with all isolated strains using raised rabbit serum against *V. anguillarum* O1 (ATCC 43305) according to Toranzo *et al.* (1987).

Bacterial cells were grown on BHA plates at 24-48 h 25°C and several loopfuls of the cells were resuspended in PBS to obtain a concentration of a Mc Farland Standart No. 3. Those whole cell suspensions we used as antigens in the slide agglutination assays. A distinct and

immediately occurring agglutination was registered as positive and weak agglutination after 5 min was considered as negative test.

Antibiotic susceptibility testing: Drug resistance patterns of the isolates were determined by the disc diffusion method on Müller-Hilton agar (Difco), using the nine antibiotics (Oxoid and bioMerieux). Incubation on Müller-Hilton agar was carried out at 21°C at 24 h.

RESULTS

Clinical findings: The disease occurred in land-based farms in the summer and water temperature was recorded as 15°C and cage farms in the autumn when water temperatures decreased to 13°C. The diseased fish showed erratic swimming, dark discoloration and exophthalmia; the mortality varied from 20 to 30% in the farms.

The external signs were haemorrhage in the anal and pectoral fins and on the abdomen and there were bilateral exophthalmia with haemorrhage in the eyes. In various parts of the body in diseased fish, especially on the flanks skin lesions as ulcers were detected. Diffuse or petechial haemorrhage on the muscle was also noted.

In internal organs, a diffuse or petechial haemorrhage in liver, pyloric caeca, peritoneal or subperitoneal membranes, perivisceral fat and lower intestines were frequent internal signs.

Challenge tests: In all, 18 out of 30 fish infected with the agent died and none of control fish died. All fish except for control group that were infected with 1.6×10^6 CFU died after 9 days. Most of fish that died had haemorrhage and necrosis in the muscle at the injection site and petechial haemorrhage in the liver, pyloric caeca and the perivisceral fat. Bacteria were isolated from 18 fish samples that died during the experiment. No bacteria were isolated from the surviving fish.

Biochemical, physiological and serological tests: The morphologic and biochemical properties of isolated *V. anguillarum* strains are presented in Table 1. Microbiological analysis of internal organs rendered in all cases the growth in pure culture of motile Gram (-) bacteria. The isolates were found to be fermentative, oxidase and catalase positive, positive for dihydrolase arginine, negative for decarboxylation of lysine and ornithine, positive for hydrolisis gelatine and sensitivity to O/129. These characteristics allowed their presumptive identification as *V. anguillarum*. In a slide agglutination test with anti *V. anguillarum* O1 (ATCC43305) serum, showed that all strains were positive.

Table 1: The morphologic and biochemical properties of isolated *V. anguillarum*

Properties	Isolates											
	1	2	3	4	5	6	7	8	9	10	11	12
Gram stain	-	-	-	-	-	-	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+
Growth in:												
0%NaCl	-	-	-	-	-	-	-	-	-	-	-	-
7%NaCl	-	-	-	-	-	-	-	-	-	-	-	-
Sensitivity to 0/129	+	+	+	+	+	+	+	+	+	+	+	+
Acid from: 0/F	+	+	+	+	+	+	+	+	+	+	+	+
ONPG	+	+	+	+	+	+	+	+	+	+	+	+
ADH	+	+	+	+	+	+	+	+	+	+	+	+
LDH	-	-	-	-	-	-	-	-	-	-	-	-
ODC	-	-	-	-	-	-	-	-	-	-	-	-
Citrate utilization	+	+	+	+	+	+	+	+	+	+	+	+
H ₂ S	-	-	-	-	-	-	-	-	-	-	-	-
Urease	-	-	-	-	-	-	-	-	-	-	-	-
TDA	-	-	-	-	-	-	-	-	-	-	-	-
Indole	-	-	-	-	-	-	-	-	-	-	-	-
VP	+	+	+	+	+	+	+	+	+	+	+	+
Gelatin	+	+	+	+	+	+	+	+	+	+	+	+
Production of asid from												
Glucose	+	+	+	+	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+	+	+	+	+
Inositol	+	+	+	+	+	+	+	+	+	+	+	+
Sorbitol	+	+	+	+	+	+	+	+	+	+	+	+
Rhamnose	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+
Melibiose	-	-	-	-	-	-	-	-	-	-	-	-
Amygdalin	-	-	-	-	-	-	-	-	-	-	-	-
Arabinose	+	+	+	+	+	+	+	+	+	+	+	+

+: Positive, -: Negative

Table 2: Antibiotic susceptibility profile of the isolates of *V. anguillarum*

Antibiotic (µg/disc)	Isolates											
	1	2	3	4	5	6	7	8	9	10	11	12
Amoxycillin (25)	S	S	S	S	S	S	S	S	S	S	S	S
Ampicillin (10)	S	S	S	S	S	S	S	S	S	S	S	S
Enrofloxacin (5)	R	R	R	R	R	S	S	S	R	R	R	S
Flumequin (5)	S	S	S	S	S	S	S	S	S	S	S	S
Furazolidone (50)	S	S	S	S	S	S	S	S	S	S	S	S
Kanamycin (30)	S	S	S	S	S	S	S	S	S	S	S	S
Oxolinic acid (30)	S	S	S	S	S	S	S	S	S	S	S	S
Oxytetracycline (30)	S	R	R	R	R	S	S	R	R	R	R	S
TMP-SDZ (25)*	S	S	S	S	S	S	S	S	S	S	S	S

S*Trimethoprim-Sulphadiazine(1/5), S: Sensitive, R: Resistant

Antimicrobial susceptibility testing: The antibiotic susceptibilities of the isolates of *V. anguillarum* from rainbow trout were assessed and are given in Table 2. Infected fish were treated with tribressen (Trimethoprim-Sulphadiazine 1/5) added to dry pellet feed (50 mg kg⁻¹ fish per day) for 10 consecutive days and mortality was completely controlled.

DISCUSSION

The first report of vibrio disease occurring in fish raised in facilities in salt water or in fresh water was given by Rucker in 1954. However, that report dealt only with vibrio disease among *Oncorhynchus gorbuscha*,

Oncorhynchus keta and *Oncorhynchus tshawytscha* raised in facilities along the Washington coast, there was no discussion of the signs or the pathogenic bacteria (Egusa, 1992). Reports on vibrio disease among salmonids have been increasing with their increased salt-water and fresh-water cultivation in Europe and the United States. Vibrosis reported that it diagnosed on *Oncorhynchus tshawytscha* in brackish water regions (Cisar and Fryer, 1969) and *Oncorhynchus gorbuscha* and *Oncorhynchus keta* in salt-water facilities (Holt, 1970; McCarthy *et al.*, 1974; Ghittino *et al.*, 1975; Egidius and Anderson, 1977). Muroga (1975) isolated *V. anguillarum* from *Salmo gairdnerii irideus* raised in salt water in Japan. Vibriosis occurs frequently among ayu

(*Plecoglossus altivelis*) fingerlings raised after capture. Muroga and Egusa (1967) have examined the bacteria isolated from diseased ayu raised in Lake Hamana and they have reported the identification of *V. anguillarum*. Eight serotypes of *V. anguillarum* are usually isolated in Japan, (Ezura *et al.*, 1980). Almost all the strains of *V. anguillarum* isolated from ayu killed by vibriosis in freshwater were serotype J-O-1 and those in seawater were serotype J-O-3. One of the characteristics of vibriosis in Lake Biwa is that mass killing of ayu have occurred not only in cultured populations but also in feral population (Eguchi *et al.*, 2000).

Vibriosis appears in mariculture facilities in Turkey, *V. anguillarum* O1, *V. ordalii* were isolated from sea bass and *V. anguillarum* O1, *V. alginolyticus* was isolated from sea bream (Çağırhan, 1993; Tanrikul *et al.*, 2005). *Yersinia ruckeri* (Çağırhan and Yürekli Türk, 1992, 1998; Tanrikul, 1995) *Lactococcus* sp. (Çağırhan and Tanrikul, 1997) and *Aeromonas salmonicida* (Kirkan *et al.*, 2003) were isolated from rainbow trout in freshwater farms. Kirkan *et al.* (2003) reported that the *Aeromonas salmonicida* was isolated from 265 rainbow trouts, which showed clinical signs from some rainbow trout farms and hatcheries in Turkey.

Vibriosis has appeared for the recently in rainbow trout farms in Turkish. Indeed, the disease in the intensive farming regions is spreading rapidly from farm to farm. On the contrary, vibrio species from salmonids were not isolated in natural streams in Japan until now (Eguchi *et al.*, 2000).

There was biochemical variability existing among *V. anguillarum* O1 strains, which were isolated from sea bass. (Tanrikul *et al.*, 2005). But from the O1 strains biochemical properties point of view, isolated *V. anguillarum* from different localities and different times in rainbow trouts did not show differences. Except fermentation of sugars, 3 isolates among the *V. anguillarum* O1 strains isolated from sea bass by (Tanrikul *et al.*, 2005) showed similarities with rainbow trout isolates from the other biochemical properties. It is thought that feed with offal, which was obtained from marine fish, has played a major role in the disease contamination in rainbow trout farms. Because, vibriosis has first appeared in some farms, where is the fingerlings fed with this type of food in hatcheries. After, contamination spreaded to the cage culture lakes by transportation. Now, the disease often appears in both fish ponds and fish cages.

In antimicrobial susceptibility tests made from isolates to become isolated from vibriosis, were found to be sensitivity of bacteria to amoxicillin, ampicillin, flumequin, furazolidone, kanamycin, oxalimic acid and

sulphonamides. Some of the isolates developed a resistance to enrofloxacin ve oxytetracycline. Because of the widespread enrofloxacin uses against the streptococcosis in rainbow trout farms in Turkey, *V. anguillarum* strains sensitivity also decreased against the chemotherapeutic (Takahashi *et al.*, 1976) recommended the sulphonamids in vibriosis treatment, (while Endo *et al.*, 1975) proposed the oxolinic acid. Although *V. anguillarum* strains were also sensitive against the sulphonamids. But it was not applied in Turkey, so that, oxytetracycline was preferred. The resistances of bacteria were gradually raised against the antibiotic by applying oxitetracycline.

Aeromonas salmonicida was not isolated in this research. However (Kirkan *et al.*, 2003) were isolated to *Aeromonas salmonicida* in the same area. So, it may be probably erroneously identification, because of the similarity of clinical symptoms of both diseases. In conclusion, in this study it is reported that *Vibrio anguillarum* O1 isolated from rainbow trout in freshwater farms of Turkey. Besides, results of antibiogram test were presented and identification of agent was supported with serologic tests.

REFERENCES

- Anderson, J. and D. Conroy, 1970. Vibrio Disease in Marine Fishes. In: A Symposium of Fishes and Shellfishes. Snieszko, F.F. (Ed.), Special Publication No. 5, American Fisheries Society, USA.
- Çağırhan, H. and O. Yürekli Türk, 1992. First isolation of *Yersinia ruckeri* from a rainbow trout farm in Turkey. In: EAAP 5th International Conference on Diseases of Fish and Shellfish 25-29 August. Budapest, Hungary.
- Çağırhan, H., 1993. An investigation on the diagnosis and the treatment of cultured sea bass and sea bream. Ph.D Thesis, Ege University, Fisheries Faculty Turkey.
- Çağırhan, H. and T.T. Tanrikul, 1997. Lactococcosis in Rainbow Trout Farm. In: Mediterranean Fisheries Congress. Hossu, B. (Ed.), E.Ü. Fisheries Faculty. Izmir, Turkey.
- Çağırhan, H. and T.T. Tanrikul, 1998. Testing the effectiveness of a *Yersinia* vaccine in infected and chemically treated juvenile rainbow trout (*Onchorhynchus mykiss*). J. Applied Ichtyol., 14: 239-243.
- Cisar, J.O. and J.L. Fryer, 1969. An epizootic of vibriosis in chinook salmon. Bull. Wild Dis. Assoc., 5: 73-76.
- Egidius, E. and K. Anderson, 1977. Norwegian references strains of *Vibrio anguillarum*. Aquaculture, 10: 215-219.

- Eguchi, M., E. Fujiwara and N. Miyamoto, 2000. Survival of *Vibrio anguillarum* in freshwater environments: Adaptation or debilitation. J. Infect. Chemother., 6: 126-129.
- Egusa, S., 1992. Infectious Diseases of Fish. Balkema, A.A. (Ed.), Publishers, Brookfield.
- Endo, T., K. Doi, H. Tanaka and Y. Watanabe, 1975. Effects of oxolinic acid on vibrio disease naturally contracted by rainbow trout. Fish Pathol., 10: 17-21.
- Ezura, Y., K. Tajima, M. Yoshimizu and T. Kimura, 1980. Studies on the taxonomy and serology of causative organisms of fish vibriosis. Fish Pathol., 14: 167-179.
- Ghittino, P., S. Andruetto and E. Vigliani, 1975. Grave vibriosis in trote iridee allevate in acqua salsa. Bull. Off. Int. Epiz., 69: 1045-1056.
- Grisez, L. and F. Ollevier, 1995. Comparative serology of the marine fish pathogen *Vibrio anguillarum*. Applied Environ. Microbiol., 61: 4367-4373.
- Holt, G., 1970. Vibriosis (*Vibrio anguillarum*) as an epizootic disease in rainbow trout (*Salmo gairdneri*). Acta Vet. Scand., 11: 600-603.
- Kirkan, S., E.Ö. Göksoy and O. Kaya, 2003. Isolation and antimicrobial susceptibility of *Aeromonas salmonicida* in rainbow trout (*Oncorhynchus mykiss*) in Turkey hatchery Farms. J. Vet. Med. Series B, 50, 7: 339-346.
- Larsen, J.L., K. Pedersen and I. Dalsgaard, 1994. *Vibrio anguillarum* serovars associated with vibriosis in fish. J. Fish Dis., 12: 259-267.
- McCarthy, D.H., J.P. Stevenson and M.S. Roberts, 1974. Vibriosis in rainbow trout. J. Wild Dis., pp: 10-17.
- Muroga, K. and S. Egusa, 1967. *Vibrio anguillarum* from an endemic disease of Ayu in Lake Hamana. Bull. Jap. Soc. Sci. Fish., 33: 636-640.
- Muroga, K., 1975. Research on *Vibrio anguillarum* and its infections. J. Fac. of Fisheries and Animal Husbandry, Hiroshima University, 14: 101-215.
- Pedersen, K., L. Grisea, R. Van Houdt, T. Tiainen, F. Ollevier and J.L. Larsen, 1999. Extended serotyping schema for *Vibrio anguillarum* with the definition and characterization of seven provision O-serogroups. Curr. Microbiol., 38: 183-189.
- Strout, R., E. Sawyer and B.A. Countermash, 1978. Pathogenic vibriosis in confinement-reared and feral fishes of the Maine-New Hampshire coast. J. Fish. Res. Board of Canada, 35: 403-488.
- Takahashi, T., S. Inoue, K. Hiramoto and K. Miyazawa, 1976. Amounts of indigestion and concentrations in tissue resulting from oral administration in *Engraulis japonica* of sulfamonomethoxine. Fish Pathol., 11: 1-4.
- Tanrikul, T.T., 1995. Vaccine application and determination of the result for protection against yersiniosis. Ph.D Thesis, Ege University, Fisheries Faculty.
- Tanrikul, T.T., H. Çağırğan and E. Toksen, 2005. The use of API 20E for the identification of isolated *Vibrio* sp. from cultured sea bass (*Dicentrarchus labrax* L.). J. Fish. Aquat. Sci., 20, 3-4: 483-490.
- Tolmasky, M.E., L.A. Actis, A. Toranzo, A. Barja and H.J. Crosa, 1985. Plasmids mediating iron uptake in *Vibrio anguillarum* strains isolated from turbot in Spain. J. General Microbiol., 131: 1989-1997.
- Toranzo, A.E., A.M. Baya, B.S. Robertson, J.L. Barja, D.J. Grimes and F.M. Hetrick, 1987. Specificity of slide agglutination test for detecting bacterial fish pathogens. Aquaculture, 61: 81-97.
- Winton, J., J. Rohovec and J. Fryer, 1983. Bacterial and Viral Diseases of Cultured Salmonids in the Pacific Northwest. In Bacterial and Viral of Fish. Crosa, J.H. (Ed.), Washington Sea Grant. Seattle, pp: 1-20.