Vagococcus salmoninarum, a causative agent of disease in rainbow trout (*Oncorhynchus mykiss*, Walbaum) broodstocks in the aegean region of Turkey

Tansel TANRIKUL[1], Meriç Lütfi AVSEVER[2], Ertan Emek ONUK[3], Behire Işıl DİDİNEN[4]

[1] Ege University, Fisheries Faculty, Fish Diseases Department, İzmir, Turkey
[2] Bornova Veterinary Control Institute, Fish Diseases National Referance Lab., Bacteriology Dept., İzmir, Turkey
[3] Ondokuz Mayıs University, Faculty of Veterinary Medicine, Dept. of Aquatic Animal Diseases, Samsun, Turkey
[4] Suleyman Demirel University, Egırdır Fisheries Faculty, East Campus, Çünür- Isparta, Turkey

Geliş Tarihi / Received: 19.11.2013, **Kabul Tarihi** / Accepted: 22.04.2014

Summary: In a commercial rainbow trout farm located in the Aegean region of Turkey, a disease outbreak with an average mortality of 10% was recorded in rainbow trout broodstocks. The disease symptoms emerged at 10-12°C water temperature after spawning periods. 6 bacterial isolates were obtained from 2 of the clinically infected rainbow trout broodstock. These isolates were observed to be Gram-positive cocobacilli and were identified phenotypically as *Vagococcus salmoninarum*. Isolates were also confirmed by using a PCR method with *V.salmoninarum* specific primers. Antibiotic susceptibility of strain VG3 was assessed by the Kirby-Bauer disk diffusion method, and the results showed that it was susceptible amoxycillin, ampicillin, enrofloxacin, norfloxacine, oxolinic acid and florfenicol. *V.salmoninarum* isolates isolated from different geographical region in Turkey were revealed to be related (0.73) as a result of ERIC-PCR.

Key words: *Oncorhynchus mykiss*, phenotypic, genotypic, characterization, *Vagococcus salmoninarum.*

Türkiye'nin Ege Bölgesindeki gökkuşağı alabalığı (*Oncorhynchus mykiss*, Walbaum) anaçlarında bir hastalık etkeni *Vagococcus salmoninarum*

Özet: Türkiye'nin Ege Bölgesi'nde yer alan ticari bir alabalık çiftliğinde gökkuşağı alabalığı anaçlarında görülen bir hastalık esnasında ortalama %10 oranında bir ölüm kaydedildi. Hastalık belirtileri yumurtlama sonrası 10-12°C su sıcaklığında görüldü. Klinik olarak enfekte 2 anaç balıktan 6 adet bakteriyel izolat elde edildi. Bu izolatların Gr (+) kokobasil oldukları tespit edildi ve fenotipik olarak *Vagococcus salmoninarum* olarak teşhis edildi. İzolatlar, aynı zamanda spesifik *V. salmoninarum* primerleri ile PCR metodu kullanılarak teyit edildi. VG3 suşunun antibiyotik duyarlılığı Kirby-Bauer disk difüzyon metodu ile belirlendi ve amoksisillin, ampisillin, enrofloksasin, norfloksasin, oksolinik asit ve florfenikole duyarlılık gösterdi. Türkiye'de farklı coğrafik bölgelerden izole edilen *V. salmoninarum* izolatlarının ERIC-PCR sonucuna göre ilişkili olduğu (0.73) görüldü.

Anahtar kelimeler: *Oncorhynchus mykiss*, fenotipik, genotipik, karakterizasyon, *Vagococcus salmoninarum.*

Introduction

Turkey's natural resources and ecological situation is very suitable for aquaculture. Turkey also has a wide variety of freshwater and marine species comprising trout, carp, sea bass, sea bream, turbot, mussel, crayfish, etc. Aquaculture is going to play an increasingly important role in the Turkish economy, as fishery products are the only products of animal origin that can be exported to the EU [17]. There has been a fast increase in the aquaculture production in Turkey with the implementation of scientific and technological modernization. The percentage of aquaculture in total fish production has been rising every year. Rainbow trout are the main cultured freshwater fish species and raceways with floating cages are employed in culture of trout [7]. Turkey has become one of the top trout producing countries in Europe with an annual production of 75 567 tonnes, or 47% of the country's total aquaculture production [4].

New and emerging fish and shellfish diseases have caused substantial economic and environmental impact in aquaculture [14]. Vagococcosis caused by *Vagococcus salmoninarum* is an emerging disease of rainbow trout in the European Union, causing mortality rates up to 50% in broodstock during the spawning period with water temperature of 10-12°C [11]. The disease was reported in

Yazışma adresi / Correspondence: *B.Işıl Didinen, Süleyman Demirel University Fisheries Faculty East Campus 32260 Çünür, Isparta, Türkiye E-posta: behiredi-dinen@hotmail.com*

rainbow trout in Australia [13], France [9], Italy [6] and Spain [11,12] and in Atlantic salmon (*Salmo salar*) and brown trout (*Salmo trutta*) in Norway. In Turkey, the only vagococcosis infection was reported from rainbow trout farms in the Mediterranean Region by Didinen et al., (2011).

Recently, a disease outbreak with mortality of 10% was observed in rainbow trout broodstocks at 12°C water temperature in post spawning periods. The diseased fish had similar clinical symptoms including darkening of skin, hemorrhages on operculum, at the base of fins, in the abdomen and around the mouth, unilateral or bilateral exophthalmia, splenomegaly, anemia in the liver and pericarditis.

Thus, we presumed that the trout might be infected by certain bacterial pathogens and we expected to isolate the pathogen from the diseased fish. In this article, we have described the isolation, phenotypic characterization of the pathogenic agent, *V. salmoninarum*, which caused infection in rainbow trout broodstock. The genetic relationship of this isolate and the *V. salmoninarum* (isolate) previously identified in Turkey was also determined.

Material and Methods

For bacteriological examinations, two moribund rainbow trout broodstock (1500-2000 g) from a commercial farm in the Aegean region of Turkey were selected. Sampling was done in March 2012 at which time the water temperature was 12°C. Samples from internal organs, kidney, spleen and liver, were streaked on trypticase soy agar (TSA) supplemented with 5% sheep blood. The plates were incubated for 48 hours at 22±1 °C. After the incubation period, colonies were selected and pure cultures were made. Isolates were identified according to their morphology, physiology and their biochemical and enzymatic properties. Conventional microbiological methods and miniaturizing systems (API 50CH and API 20 STREP) were used for phenotypical characterization of the isolates. Molecular identification of isolates was carried out specific PCR using the oligonucleotide primers of with *V. salmoninarum* pSal-1 (5'-GTTTTAGCCGCATGGCTGAGATAT-3') and pSal-2(5'AGGTGGGAACAGTTACTCTCCCA-3) (Ruiz-Zarzuela et al., 2005). A 25 µl PCR mastermix containing DEPC-treated water, 1xPCR Buffer, 1.5 mM of MgCl2, 0.2 mM of each dNTP's, 1.0 U Taq polymerase, 100 pmol of each primer and 5 µl template DNA was used. Amplification started with an initial denaturation step of 3 min. at 94°C. It was followed by 35 cycles which consisted of a denaturation step for 1 min. at 94°C, primer annealing at 55°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 10 min. *V. salmoninarum* NCIMB 13133 and Lactococcus garviae ATCC 43921 reference strains were used as a positive and negative control respectively.

A representative isolate VG3 isolated from kidney of the 6 similar field isolates was tested for its antibiotic-resistance by the Kirby-Bauer disk diffusion method on Mueller-Hinton agar (Oxoid). The antibiotics (Oxoid) were tested including amoxicillin (30 µg), ampicillin (10), enrofloxacin (5 µg), flumequin (30 µg), erythromycin (15 µg), florfenicol (30 µg), norfloxacin (2 µg), oxolinic acid (2 µg), oxytetracycline (30 µg) and trimethoprim-sulphadiazine (25 µg). NCCLS standards were used for the evaluation of the results (NCCLS, 2001).

The clonal relationship among the Turkey isolates and the reference strain was determined with ERIC-PCR using the ERIC-2 primer (5'-AAGTAAGTGACTGGGGTGAGCG–3') which is specific for Enterobacterial repetitive intergenic consensus (ERIC) sequences (Versalovic et al., 1991). The DNA profiles were analyzed with CHEF-DR® III, Quantity One® software (Bio-Rad Laboratories, Hercules, CA). The dendogram analysis was performed according to unweighted-pair group method (UPGMA). The DNA profiles were analyzed with CHEF-DR® III, Quantity One® software (Bio-Rad Laboratories, Hercules, CA). The dendogram analysis was performed according to unweighted-pair group method (UPGMA).

Findings

Only one type of colony was observed after 48 hours of incubation of each plate. Overall 6 isolates (VG1 to VG6) isolated from the liver, kidney and spleen were restreaked on TSA agar in order to obtain stock cultures. All isolates, which showed similar phenotypical characteristics, were detected as *V. salmoninarum* in conventional microbiological methods and API systems. The phenotypic characteristics of *V. salmoninarum* isolates are presented in Table 1 on the basis of conventional methods and

API 20 STREP miniaturized systems. Furthermore, all *V. salmoninarum* field isolates were confirmed by PCR assay using specific primers, giving the expected amplification product of 300 bp (Fig. 1).

Table 1. Phenotypical characterization of *V. salmoninarum* isolates.

Test methods	*V. salmoninarum* isolates (VG1- VG6)				
	Characteristics	Reactions	Test methods	Characteristics	Reactions
Microscope	Gram stain	+	API 50 CH	Glycerol	-
	Motility	-		Erythritol	-
	Shape	Coccobacilli		D-Arabinose	-
Conventional	Catalase	-		L-Arabinose	-
Methods	Oxidase	-		D-Ribose	+
	Haemolysis	α		D-Xylose	-
	Aesculin hydrolysis	+		L-Xylose	-
Growth in NaCl	6,50%	-		D-Adonitol	-
Growth at	10 °C	+		Methy-βD-xylopyranoside	-
	20 °C	+		D-Galactose	-
	37 °C	+		D-Glucose	+
	42 °C	-		D-Fructose	+
Growth on McConkey agar		-		D-Mannose	+
	Sensitivity to O/129	+		L-Sorbose	-
	Urease	-		L-Rhamnose	-
	Indole	-		Dulcitol	-
	Acid from:O/F	F		Inositol	-
	ONPG	-		D-Mannitol	
	ADH	-		D-Sorbitol	-
	LDH	-		Methyl-αD-Mannopyranoside	-
	ODC	-		Methyl- αD-Glucopyranoside	-
	Nitrate reduction	-		N-Acetyl glucosamine	+
	H_2S	+		Salicin	+
Acid from	Glucose	+		D-Cellobiose	+
	Mannitol	+		D-Maltose	+
	Inositol	+		D-Lactose	-
	Sorbitol	+		D-Melibiose	-
	Rhamnose	-		D-Saccharose	-
	Sucrose	+		D-Trehalose	+
	Melibiose	-		Inuline	-
	Amygdalin	-		D-Melezitose	-
	Arabinose	+		D-Raffinose	-
API 20 STREP	Lactose	-		Amidon	-
	Trehalose	+		Glycogene	-
	Inuline	-		Xylitol	-
	Raffinose	-		Gentiobiose	+
	Amygdalin	+		D-Turanose	-
	Glycogen	-		D-Lyxose	-
	β-Haemolysis	-		D-Tagatose	+
	Arbutin	+		D-Fucose	-
				L-Fucose	-
				D-Arabitol	-
				L-Arabitol	-
				Potasium Gluconate	-
				Potasium 2- Ketogluconate	-
				Potasium 5 -Ketogluconate	-

+:Positive, -:Negative.

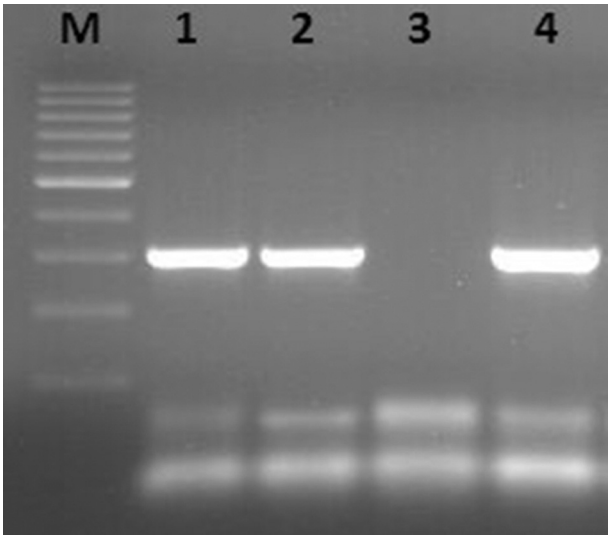

Fig. 1. *V. salmoninarum* specific PCR, 300 bp. M; 100-1000 bp DNA ladder, **1:** *V. salmoninarum* NCIMB 13133, **2:** *V. salmoninarum* field isolate (Aegean region), **3:** *Lactococcus garviae* ATCC 43921, **4:** *V. salmoninarum* field isolate (Mediterranean region).

ERIC-PCR revealed different genotypic profiles for two Turkey isolates and the reference V. salmoninarum strain. The isolates were grouped under a unique type and a cluster according to 70% similarity coefficient index. Isolates obtained from Turkey were found to be related to each other (0.73) (Fig. 2).

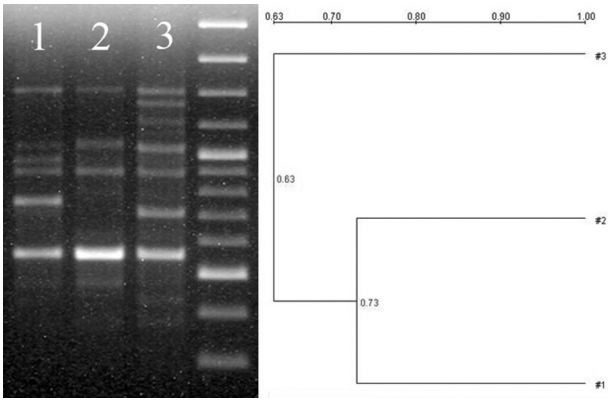

Fig. 2. Genotypic profiles and phylogenetic tree obtained with ERIC PCR **1:** *V. salmoninarum* field isolate (Aegean region), **2:** *V. salmoninarum* field isolate (Mediterranean region), **3:** *V. salmoninarum* NCIMB 13133.

As a result of the antimicrobial sensitivity test with the representative isolate, sensitive to amoxicillin, ampicillin, enrofloxacin, norfloxacin, oxolinic acid and florfenicol and resistant to flumequin, erythromycin, oxytetracycline and trimethoprim-sulphadiazine was observed. The treatment was carried out orally with florfenicol (50 mg/kg body weight) supplemented feed for 10 days. Also, eggs obtained from infected fish were seen to hatch.

Discussion and Conclucsion

Streptococcosis in fish are defined as a disease complex caused by a few Gram-positive cocci belonging to different genera and species [15]. These infections are classified under 2 groups etiologically such as warm water and cold-water streptococcosis [11,15]. Cold-water streptococcosis is reported to be less widespread than warm water streptococcosis. Cold water streptococcosis caused by *V. salmoninarum* is an emerging disease of rainbow trout industry in European Union [11]. In Turkey, the only isolation of agent was accomplished in 2011 from a commercial rainbow trout farm in the Mediterranean Region [5]. They reported that the disease has an approximate mortality rate of 55% in the two-month period. Therefore, *V. salmoninarum* is very important pathogen for rainbow trout. In the present study, the first isolation of *V. salmoninarum* in the Aegean region, shows that the disease spreads to different geographic regions and will gain importance in the future.

Diseased rainbow trout showed darkening of skin, hemorrhages on operculum, at the base of fins, in the abdomen and around the mouth, unilateral or bilateral exophthalmia, splenomegaly, anemia in the liver and pericarditis. Clinical findings were similar to those previously described by Michel et al., 1997, Ruiz-Zarzuela et al., 2005, Didinen et al., 2011. Varying mortality rates observed in different cases are thought to be related to stress factors such as population density, low water quality, photoperiod applications [5]. In our study, the infection has mortality at level of 10%, which was found to be lower than the rates suggested by previous reports in France and Turkey [5,8]. Ruiz-Zarzuela et al., (2005) reported the mortality rates in rainbow trout

were between 11-36% during 1999-2001 in Spain. This finding was similar to the present study.

Classical microbiological methods and rapid diagnostic kits such as API 20 STREP and API 50 CH are used for the identification of *V.salmoniarum* isolates [5,8,11]. Phenotypical characterization study has revealed that these isolates have many common biochemical properties but some characteristics show variations among strains. Previous reports have shown that acid production from ribose, sorbitol, L-sorbose, cellobiose, maltose, β-gentiobiose and L-fucose vary among strains [8]. Also, Ruiz-Zarzuela et al., (2005) have declared that catalase, H2S production, growth on MacConkey agar, hippurate hydrolysis, pyrolidonyl arylamidase, α-galactosidase and alkaline phosphatase enzyme production, and acid production from mannitol, sorbitol, lactose, L-arabinose, D-xylose, L-sorbese, rhamnose, inositol, saccharose and melecytose vary among different strains. The *V. salmoninarum* isolates in this study are observed to have common phenotypical characteristics and these characteristics are found to be similar with other researchers' findings [8,11] When compared to the *V. salmoninarum* isolates from the previous study [5], differences in H2S production and in acid production from maltose, saccharose and β-gentibiose were observed. Also, the ability of growth at 37 °C of *V. salmoninarum* isolates in this study is found to similar to reports by Didinen et al. (2011) and Michel et al., (1997), but different from those of Ruiz-Zarzuela et al. (2005).

V. salmoninarum's resistance to sulphonamides has been reported in the previous studies [5,8,11]. *V. salmoninarum* were also resistant to trimethoprim-sulphadiazine in this study. In addition, Didinen et al., (2011) noted that *V. salmoninarum* showed resistance to oxytetracycline. The same result was also observed in this study.

In the current study *V. salmoninarum* isolates were sensitive to amoxicillin, ampicillin, enrofloxacin, norfloxacin, oxolinic acid and florfenicol. Didinen et al., (2011) also reported similar results with respect to amoxicillin, ampicillin, enrofloxacin and florfenicol. In contrast, Ruiz-Zarzuela et al., (2005) noted that the majority of *V. salmoninarum* strains were resistant to amoxicillin and ampicillin.

Ruiz-Zarzuela et al., (2005) have declared that erythromycin and oxytetracycline are effective only in short term treatments that last for 5-7 days and that mortality rate can be lowered after prolonged periods of treatment which can lead to antibiotic resistance. Didinen et al., (2011) reported that *V. salmoninarum* isolates were susceptible to erythromycin and doxycycline but these antibiotics were found to be in effective during treatment. In this work, florfenicol was found to be effective in vitro and in vivo.

Molecular typing is a powerful tool in determining the clonal relations between isolates obtained from different hosts or environment and it provides evidence on common infection routes of pathogenic agents. In the epidemiological analyses of bacteria, several genotyping strategies such as restriction fragment length polymorphism (RFLP), pulsed-field gel electrophoresis (PFGE), randomly amplified polymorphic DNA analysis (RAPD) and repetitive-sequence-based polymerase chain reaction (Rep-PCR) have been used. PFGE is considered to be the best method in typing bacteria but technical hardships and its laborious process create limitations for routine applications. RAPD and Rep-PCR are fast and easily applicable methods when compared to PFGE [3]. Lately, ERIC PCR has become popular in determining the genetic relations between bacterial fish pathogens [1,3,10]. In this study, genotyping of *V. salmoninarum* isolates with the ERIC PCR method using the ERIC2 primer was achieved and epidemiological application of this method was found to be possible. Although isolates from Turkey were found to be closely related, genotypic analyses of isolates from other countries are necessary for determining whether these isolates are native.

The mortality levels of vagococcosis cases are observed to rise as fish are exposed to stress factors [8]. This fact emphasizes the importance of animal welfare and good husbandry. For this reason, optimum conditions have to be provided during fish production. As *V. salmoninarum* has the potential to be an emerging fish pathogen, vaccine applications will gain importance in the future for prevention and control purposes.

References

1. **Altun S., Onuk EE, Çiftci A, Büyükekiz AG, Duman M,** (2012). *Phenotypic, Genotypic Characterization and Antimicrobial Susceptibility Determination of Lactoccous garvieae Strains*. Kafkas Univ Vet Fak Derg. 19(3), 375-381.
2. **Austin B, Austin DA** (2007). *Bacterial Fish Pathogens: Diseases of Farmed and Wild Fish*, 4th edition, Springer-Praxis, Chichester, UK, p.552.
3. **Beaz-Hidalgo R, Lopez-Romalde S, Toranzo AE, Romalde JL,** (2008). *Polymerase Chain Reaction Amplification of Repetitive Intergenic Consensus and Repetitive Extragenic Palindromic Sequences for Molecular Typing of Pseudomonas anguilliseptica and Aeromonas salmonicida.* J Aqua Anim Health. 20, 75-85.
4. **FAO.** *National Aquaculture Sector Overview Turkey.* Fisheries and Aquaculture Department. Erişim adresi: http://www.fao.org/fishery/countrysector/naso_turkey/en#tcN90158, Erişim tarihi:2013
5. **Didinen BI, Kubilay A, Diler, O, Ekici S, Onuk EE, Findik A,** (2011*). First isolation of Vagococcus salmoninarum from cultured rainbow trout (Oncorhynchus mykiss, Walbaum) broodstocks in Turkey.* Bull Eur Ass Fish Pathol. 31(6), 235-243.
6. **Ghittino, C, Agnetti, F, Panzieri, C, Cabra, S, Colussi, S, Prearo M,** (2004*). L'infezione da Vagococcus salmoninarum della trota iridea in Italia*. Ittiopatologia 1, 25-33.
7. **Harlioglu, AG,** (2011*). Present status of fisheries in Turkey.* Rev Fish Biol Fisher. 21, 667-680.
8. **Michel, C., Nougayrede, P., Eldar, A., Sochon, E., de Kinkelin P,** (1997). *Vagococcus salmoninarum, a bacterium of pathological significance in rainbow trout Oncorhynchus mykiss farming. Dis Aquat Organ.* 30, 199-208.
9. **NCCLS,** (2001). *National Committee for Clinical Laboratory Standards of Antimicrobial Susceptibility Testing. Performance Standards for Antimicrobial Susceptibility Testing.* Eleventh Information Supplement. NCCLS document M100-S11 NCCLS, West Walley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA.
10. **Onuk, EE, Çiftçi, A, Fındık, A, Çiftçi, G, Altun, S, Balta, F, Ozer, S, Coban AY,** (2011). *Phenotypic and Molecular Characterization of Yersinia ruckeri Isolates from Rainbow Trout (Oncorhynchus mykiss, Walbaum, 1792) in Turkey.* Berl Munch Tierarztl Wochensch. 124 (7-8), 320-328.
11. **Ruiz-Zarzuela, I, de Blas, I, Girones, O, Ghittino, C, Muzquiz JL,** (2005). *Isolation of Vagococcus salmoninarum in rainbow trout Oncorhynchus mykiss (Walbaum), broodstocks: characterisation of the pathogen.* Vet Res Commun. 29, 553-562.
12. **Salogni, C, Perantoni, P, Pitozzi, A, Loris, G, Alborali GL,** (2007). *Vagococcus salmoninarum: descrizione di un focolaio di malattia in riproduttori di trota iridea (Oncorhynchus mykiss).* Ittiopatologia 4, 59-66.
13. **Schmidtke, LN and Carson J,** (1994). *Characteristics of Vagococcus salmoninarum isolated from diseased salmonid fish.* J Appl Bacteriol. 77, 229-236.
14. **Thrush, MA, Dunn, PL, Peeler EJ,** (2012). *Monitoring Emerging Diseases of Fish and Shellfish Using Electronic Sources.* Transbound Emerg Dis. 59, 385-394.
15. **Toranzo, AE, Magarinos, B., Romalde JL,** (2005). *A review of the main bacterial fish diseases in mariculture systems. Aquaculture* 246, 37-61.
16. **Versalovic, J, Koeuth, T, Lupski R,** (1991). *Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes.* Nucleic Acids Res. 19(24), 6823-6831.
17. **Yılmaz, S, Akay, AS, Gumus E,** (2008). *Fisheries sector in Turkish economy and marketing of fishery products.* Akdeniz Univ. Ziraat Fak. Derg. 21, 265-272.