JOURNAL OF PURE AND APPLIED MICROBIOLOGY, May 2014. Vol. **8**(Spl. Edn. 1), p. 119-124

Phenotypic, Histopathologic and Genomic Diagnosis of A Novel Fish Pathogen *Vagococcus salmoninarum* in Turkey

Ifakat Tulay Cagatay[1]* and Erkan Gumus[2]

[1]Akdeniz University, Faculty of Fisheries, Department of Basic Sciences, Molecular Microbiology Section, Campus, 07058, Antalya, Turkey.
[2]Akdeniz University, Faculty of Fisheries, Department of Aquaculture, Campus, 07058, Antalya, Turkey.

(Received: 28 January 2014; accepted: 06 March 2014)

In the present study, the aim was to diagnose *Vagococcus salmoninarum* by using phenotypic, histopathologic and genomic methods together. Vagococcosis is a novel and emerging disease in rainbow trout (*Oncorhynchus mykiss*) farms and outbreak of the disease results in considerable economic losses to the aquaculture industry worldwide. The sixty bacterial isolates from fish tissues which had widespread infection were identified and characterized as *V. salmoninarum* by studying the cultural pattern and biochemical-enzymatic traits with BBL Crystal™ identification system. Among sixty phenotypical positive isolates, only 30% percent were verified as *V. salmoninarum* by PCR amplification of bacterial 16S rDNA gene. Further, observed spots of bacterial accumulation in histological sections of heart and kidney were subsequently diagnosed as *V. salmoninarum* with 16S rDNA gene amplification.

Key words: *Vagococcus salmoninarum*, Rainbow trout, PCR, histopathology.

Besides several different Gram-positive coccal diseases in Rainbow trout (*Oncorhynchus mykiss*), the cold water 'streptococcosis' known as 'Vagococcosis' caused by *V. salmoninarum* can also be detrimental for aquaculture industry[1,2]. The causative agent bacterium is chain-forming, nonmotile and slow growing pathogen which occurs at temperature below 15 ^{o}C[3,4]. Vagococcosis of rainbow trout was first observed in USA in 1990[3] and in the following years, a number of outbreaks of the disease have been seen in Europe[5,6,7]. The disease is responsible for mortality rates of 20-50 % in adult trout during the spawning period[6,8]. Some previous studies showed that Streptococcosis associated pathogens including Vagoccoci were identified by clinical signs, culture-based methods, biochemical tests or histological observations. Nevertheless, differentiate of these bacteria could be difficult because of their similar physicological and histopathological characteristic[9,4]. Because of this, so far there have been only a few studies reported about *V. salmoninarum* in different regions of the world[5,10,11] and also no reports were found in literature related to the prevalence and pathogenicity of the disease. Wallbanks *et al*. (1990) differentiated the phylogenetic relationship of the genus of Vagococcus by using reverse transcriptase sequencing of 16S rRNA from diseased salmonid fish for the first time. Michel *et al.* (1997) provided some clinical, microscopical and DNA related data of Vagococcus which were responsible from heavy mortality occurred in France. Ruiz-Zarzuela *et al.*

* To whom all correspondence should be addressed.
Tel.: +90 242 3106089; Fax. + 90 242 2262013
E-mail. tulaycagatay@akdeniz.edu.tr

(2005) has demonstrated *V. salmoninarum* isolates with characterization of their cultural and biochemical traits from broodstock rearing in Spain. It has been showed that Italian, Spanish and France strains of *V. salmoninarum* were resistant to most of the registered antibiotics in European Union[12]. Therefore, the identification and diagnosis of *V. salmoninarum* and the effective control of the disease are very crucial and important to prevent economic losses for a country like Turkey which is the third biggest rainbow trout producer in Europe with her production capacity of 110 thousand tons in 1500 farms[13].

Aim of present study was to diagnose of *V. salmoninarum* from direct fish tissues, wax-embedded tissues and bacterial isolates from fish by using three diagnostic methods together during outbreak periods that occur in the South Part of Turkish trout farms with a water temperature range between 10-15 °C.

MATERIALS AND METHODS

Sample collection

From 2011 to 2013, total of 300 dead rainbow trout which had Vagococcus infection (average body weight 200-250 g) were collected from South Part of Turkey. Necropsies of fish for microbiologic and histopathologic analysis were made *in situ*.

Microbiologic characterization

Bacterial samples from kidney, spleen, liver, gill and heart from each symptomatic fish were taken by using sterile swabs. The swabs immediately streaked onto trypticase soy agar (TSA) (BD, USA) and then incubated at 15 °C for 48-72 h[14]. Isolated colonies were sub-cultured twice and were biochemically characterized with BBL Crystal™ GP identification system (BD, Becton Dickinson, USA) by manufacturer's instructions and further they were compared with characteristics in reference strain of *V. salmoninarum* ATCC® 51200 which was obtained from American Type Culture Collection (ATCC, USA). Stock cultures were maintained in a broth medium supplemented with glycerol at -20 °C.

Histologic examination

Each collected tissue (0.1-0.5 g) was washed with physiological saline solution (0.75% NaCl) and immediately fixed in 10% buffered formalin solution for histopathology[15,16]. Tissues were then processed using Hibiya's[17] fish histology method and 4 µm thick sections were stained with hematoxylin-eosin. Histological slides were evaluated on an Olympus AX-70 photomicrographic system (USA).

Genomic identification

Bacterial DNAs were extracted from four different materials: 1) from direct tissues, 2) from wax-embedded tissues, 3) from culturated bacterial isolates from tissues 4) from reference strain.

Bacterial DNA from direct tissues was extracted and purified by using DNeasy Tissue Kit (Qiagen, USA) following manufacturer's instructions. Bacterial DNA from all wax-embedded tissues was extracted by the modified method of Crumlish *et al*.[18]. 4 µm thick wax-embedded tissues sections were cut using a microtome and 5-6 tissue sections were placed into sterile 2 mL tubes. The sections were de-waxed in 1.5 mL xylene, vortexed and centrifugated at 10.000x*g* for 5 min, and the xylene removed and replaced with absolute ethanol (1.5 mL). The xylene/alcohol washing was repeated and pellets were kept for DNA purification by using DNeasy Tissue Kit (Qiagen, USA) following manufacturer's instructions. Genomic DNAs from the cultured isolates and reference bacteria were purified by using DNA Extraction Kit (Qiagen, USA) as per the manufacturer's instructions with minor modifications. Briefly, 1.5 mL of bacterial suspension which was grown in trypticase soy broth (BD, USA) were transferred to 2 mL centrifuge tubes, and centrifuged at 9000x*g* for 5 min. Supernatants were discarded and the pellets were dissolved in 1 mL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). This solution was used for DNA extraction. All purified DNA were stored at -20 °C until use.

The PCR amplification was performed out in the thermocycler (Techne, USA) by using a pair of 16S rDNA-rev primer (5'-GTTTTAGCCGCA TGGCTGAGATAT-3') and 16S rDNA-fwd primer (5'-AGGTGGGAACAGTTACTCTCCCA-3')[6]. Reaction was carried out in a volume of 25 µL containing 5 µL of the DNA, 1U of Taq polymerase, 1 µM of each primer, 0.50 mM of each dNTP, 2.5 mM $MgCl_2$. The amplification was carried out the following conditions: an initial heat denaturation at 94 °C for 5 min followed by 36 cycles of heat denaturation at 94 °C for 2 min, 55 °C for 1 min and

72 °C for 1 min. After last cycle, samples were maintained at 72 °C for 10 min for final extension. PCR products were analyzed by agarose electrophoresis (Applichem, USA). DNA bands were visualized over geldoc system (DNR geldoc, USA).

RESULTS AND DISCUSSION

Total of 300 diseased fish were obtained over the period of 2011 to 2013. Affected trout displayed external signs such as lethargy, erratic swimming, anorexia, hemorrhage, dark pigmentation, and exophthalmos in ocular area in only heavily infected fish. At necropsy of our samples, congestion and hemorrhages in gills and in the organs were noted (Fig. 1c). Additionally, internal lesion and paleness were observed in gill, heart and kidney (Fig. 1a, 1b, 1e, 1f). Other macroscopic internal signs were enlargement in spleen and liver (Fig. 1a, 1d), acids in abdomen area (Fig. 1d), pericarditis and congestion on heart and vessels (Fig. 1d, 1e). General clinical signs of our fish were very similar to results of Chang and Plumb[19] and Muzquiz *et al.*[9].

The 300 swabs from kidney, spleen, liver, gill and heart of each symptomatic fish were streaked onto TSA (BD, USA) plates and then inoculated for isolation, identification and diagnosis of the bacteria. Observed colonies on the inoculated TSA plates were usually opaque and circular with diameter of 0.5 to 1.0 mm. Phenotypic traits of all bacterial isolates were characterized and compared with reference strain

Table 1. Biochemical and enzymatic characteristics of field isolates as *Vagococcus salmoninarum* on the BBL Crystal™ GP identification system (+: positive, -: negative, NA: not available)

Characteristics	Isolate 1 from Heart	Isolate 2 from Kidney	*V. salmoninarum* ATCC 51200
Growth at			
10 °C	+	+	+
15 °C	+	+	+
20-22 °C	+	+	+
Hemolysis	α	α	α
Growth in			
2 % NaCl	+	+	+
4 % NaCl	+	+	+
Arabinose	-	-	-
Mannose	+	+	+
Sucrose	+	+	+
Melibiose	-	-	-
Rhamnose	-	-	-
Sorbitol	-	-	-
Mannitol	-	-	-
Adonitol	-	-	-
Galactose	-	-	-
Inositol	-	-	-
Esculin	+	+	+
Urea	-	-	-
Glycine	+	+	+
Treazolium	+	+	+
Arginine	-	-	-
Lysine	NA	NA	NA
p-nitrophenyl galactosidase	-	-	-
p-nitrophenyl α–β-glucosidase	+	+	+

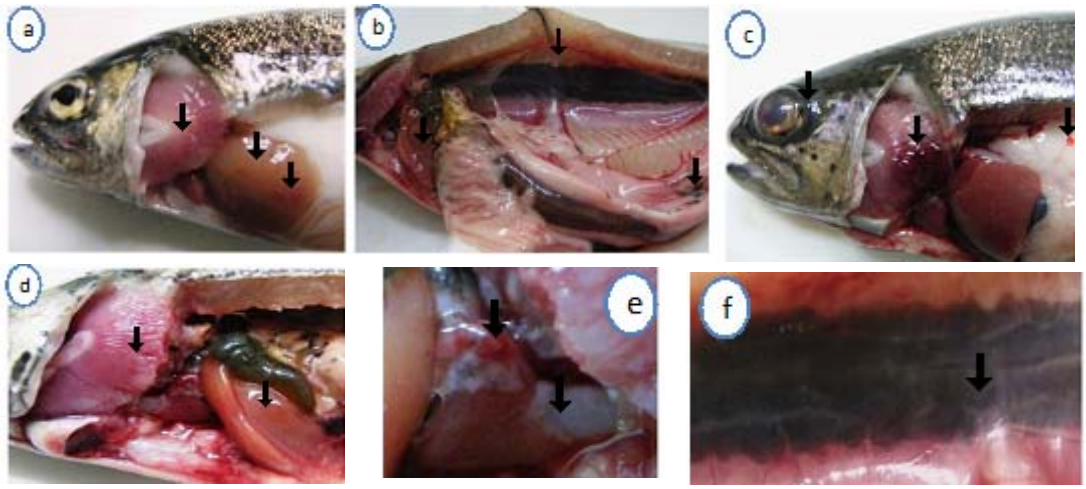

Fig. 1 (a-d) Arrows show severe infected areas in rainbow trout e) *V. salmoninarum* infection on heart f) *V. salmoninarum* infection on kidney

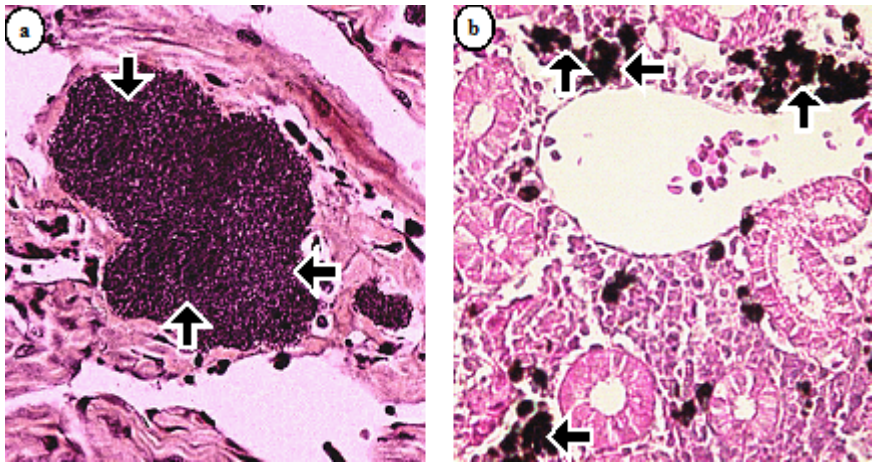

Fig. 2 (a). Histologic lesion of heart tissue (ventricle) with massive *V. salmoninarum* infection, b) Histologic lesion of kidney tissue with light *V. salmoninarum* infection (Arrows show bacterial infection spots) (Hematoxylin and eosin stain x100)

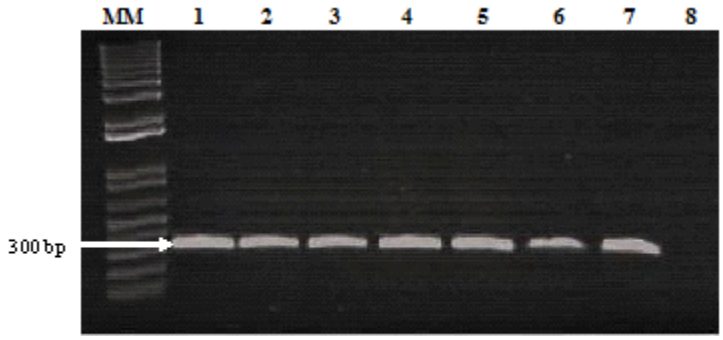

Fig. 3. Agarose gel electrophoresis of 16S rDNA gene amplification of *V. salmoninarum*. MM. Molecular marker, 1. Reference strain as positive control 2. Direct heart tissue sample 3. Wax-embedded heart tissue 4. Bacterial isolate from heart 5. Direct kidney tissue sample 6. Wax-embedded kidney tissue 7. Bacterial isolate from kidney 8. Negative control.

V. salmoninarum ATCC® 51200 (ATCC, USA) based on their biochemical and enzymatic profiles by using commercial BBL Crystal™ GP identification system (BD, USA). Our phenotypic identification results showed that sixty isolates were positive for *V. salmoninarum*. Table 1 shows the confirmation of characteristics of isolate 1 from heart and isolate 2 from kidney as *V. salmoninarum* and their validations with reference strains. None of the bacterial isolates from gills, liver and spleen tissues were identified and diagnosed as positive for *V. salmoninarum*. The study of Shewmaker *et al.*[20] has shown that *V. salmoninarum* were placed in member of streptoccocus group IV, often difficult to differentiate based on the morphological and phenotypic characters with manual or commercial systems. Although, it was not easy to show phenotypic discrimination between isolates, our results were consisted with previous characterization studies[6,8,10].

A total of 60 tissues which showed macroscopic sign of infection were used for the histopathologic analysis. The histologic observation in the heart was generally showed severe congestion and mild haemorrhages (Fig. 2a). The histologic section of kidney showed diffuse necrosis of the haematopoietic tissue and mild to moderate cellular debris (Fig. 2b). Ten heart and six kidney tissue sections that were exhibited bacterial colonies were later diagnosed as positive for *V. salmoninarum* with 16S rDNA gene analysis. The dark zones in heart and kidney tissue sections indicated by arrows in both Fig. 2a and 2b illustrated severe infection areas of coccoidal *V. salmoninarum*. No bacterial accumulation was visualized in histological section of gills, spleen and liver and the heart and the kidney were the targeted organs with heavily pathologic inflammation. Earlier report of Michel *et al.*[10] was supported our findings by indicating massive infection on the epicardium and in the blood capillaries of fish. Even though hyperaemia and oedema were present in most tissues, no bacterial spots of *V. salmoninarum* were demonstrated in spleen, gills or liver tissue sections. In many studies indicate that histopathology is known as efficient method to prove of infection[21,22], we have shown that it was inadequate to identify specify of bacteria that seen in the tissue-sections. To eliminate this inadequacy in histopathologic sections, bacterial DNAs amplification from direct and wax-embedded tissues were eventually used to diagnose of *V. salmoninarum* by PCR.

The specific 16S rDNA gene was used for diagnosis of *V. salmoninarum* from direct tissues, wax-embedded tissues and bacterial isolates. PCR results from those samples showed positive amplification products for *V. salmoninarum* at the size of 300 bp (Fig. 3). The PCR results of our bacterial isolates were confirmed with *V. salmoninarum* ATCC® 51200 reference strain. No PCR products were detected in any of other tissues such as gills, liver and spleen, or other isolates and in the negative control. The PCR results of 16S rDNA amplification products (300 bp) of Turkish *V. salmoninarum* isolates were similar to the first report of Vagococcus by Wallbank *et al.*[3].

Consequently, in this study, the complete diagnosis of *V. salmoninarum* from rainbow trout were proven successfully on firstly by observing clinical signs, identification and characterization on basis of the phenotypic and histopathologic traits and then subsequently the detection of 16S rDNA gene from the target bacteria.

ACKNOWLEDGMENTS

A part of this research was supported by the Scientific Research Projects Coordination Unit of Akdeniz University (2012.01.0111.001). We are grateful to Prof. Dr. Dürdane Kolankaya and Msc student Eylül Turasan from Hacettepe University, Biology Department for their technical assistance on histology studies.

REFERENCES

1. Toranzo, A.E., Devesa, S., Heinen, P., Riaza, A., Nubez, S. and Baja, J.L. Streptococcosis in cultured turbot caused by an Enterococcus-like bacterium. *Bull. Euro. Assoc. Fish Pathol.*, 1994; **14**: 19-23.
2. Pridgeon, J.W. and Klesius, P.H. Major bacterial diseases in aquaculture and their vaccine development. *CAB Rev.*, 2012; **7**: 1-16.
3. Wallbanks, S., Martinez-Murcia, A.J., Fryer, J.L., Phillips, B.A. and Collins, M.D. 16S rRNA sequence determination for members of the genus *Carnobacterium* and related lactic acid bacteria and description of *Vagococcus salmoninarum* sp. nov. *Inter. Syst. Bacteriol.* 1990; **40**: 224-

230.
4. Mata, A.I., Gibello, A., Casamayor, A., Blanco, M.M., Domínguez, L. and Fernández-Garayzábal, J.F. Multiplex PCR assay for detection of bacterial pathogens associated with warm-water Streptococcosis in fish. *Appl. Environ. Microbiol.*, 2004; **70**: 3183-3187.
5. Schmidtke, L.M. and Carson, J. Characteristics of *Vagococcus salmoninarum* isolated from diseased salmonid fish. *J. Appl. Bac.*, 1994; 77: 229-236.
6. Ruiz-Zarzuela, I., de Blas, I., Girones, O., Ghitino, C. and Mùzquiz, J.L. Isolation of *Vagococcus salmoninarum* in rainbow trout *Oncorhynchus mykiss* (Walbaum), broodstocks: characterisation of the pathogen. *Vet. Res. Comm.*, 2005; **29**: 553-562.
7. Ghittino, C., Agnetti, F., Panzieri, C., Cabra, S., Colussi, S. and Prearo, M. L'infezione da *Vagococcus salmoninarum* della trota iridea in Italia. *Ittiopatologia,* 2004; **1**: 25-33.
8. Salogni, C., Perantoni, P., Pitozzi, A., Loris, G. and Alborali, G.L. *Vagococcus salmoninarum:* descrizione di un focolaio di malattia in riproduttori di trota iridea (*Oncorhynchus mykiss*). *Itiopatologia,* 2007; **4**: 59-66.
9. Múzquiz, J.L., Royo, F.M., Ortega, C., De Blas, I., Ruiz, I. and Alonso, J.L. Pathogenicity of streptococcosis in rainbow trout (*Oncorhynchus mykiss*): Dependence on age of diseased fish**.** *Bull. Euro. Assoc. Fish Pathol.*, 1999; **19**: 114-119.
10. Michel, C., Nougayrede, P., Eldar, A., Sochon, E. and de Kinkelin, P. *Vagococcus salmoninarum,* a bacterium of pathological significance in rainbow trout *Oncorhynchus mykiss* farming. *Dis. Aqua. Org.*, 1997; **30**: 199-208.
11. Ghittino, C. and Pedroni, A. Main bacterial pathologies in salmonid fish: Diagnosis, therapy and prevention. In: Baruchelli, G. (Eds) *Modern Trout Farming, ESAT News,* 2001; **17**: 41-56.
12. CEC, Council of the European Communities. Council regulation no 2377/90 EEC laying down a community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. *Offic. J. Euro. Commun.*, L224,1990; pp 1-8.
13. TFS, *Turkish Fishery Statistics.* Agricultural and Rural Affairs Ministry. Ankara, 2012, Turkey.
14. Austin, B. and Austin, D.A. Bacterial Fish Pathogens: Diseases of Farmed and Wild Fish, 4thed. Springer-Praxis, Chichester, 2007, UK.
15. Hossain, M.K., Hossain, M.D. and Rahman, M.H. Histopathology of some diseased fishes. *J. Life Earth Sci.*, 2007; **2**: 47-50.
16. Ferguson, H.W. Kidney *In*: Systemic pathology of fish, Scotia Press, London, UK. 2006; pp 91-118.
17. Hibiya, T. An Atlas of Fish Histology-Normal and Pathological Features, Lubrecht and Cramer Ltd, 1995; New York.
18. Crumlish, A., Diab, M., George, S. and Ferguson, H.W. Detection of the bacterium *Flavobacterium psychrophilum* from a natural infection in rainbow trout, *Oncorhynchus mykiss* (Walbaum), using formalin-fixed, wax-embedded fish tissues. *J. Fish Dis.*, 2007; **30**: 37-41.
19. Chang, P.H. and Plumb, J.A. Histopathology of experimental Streptococcus sp. Infection inTilapia, *Oreochromis niloticus* and channel catfish, *Ictalurus punctatus* (Rafinesque). *J. Fish Dis.*, 1996; **19**: 235-241.
20. Shewmaker, P.L., Steigerwalt, A.G., Morey, E.R., Carvalho, M.G.S., Elliott, J.A., Joyce, K., Barrett, T.J., Teixeira, L.M. and Facklam, R.R. *Vagococcus carniphilus* sp. nov., isolated from ground beef. *Inter. J. Sys. Evo. Microbiol.*, 2004; **54**: 1505–1510.
21. Bernet, D., Schmidt, H., Meier, W., Burkhardt-Holm, P. and Wahli, T. Histopathology in fish: proposal for a protocol to assess aquatic pollution. *J. Fish Dis.*, 1999; **22**: 25-34.
22. Procop, G.G. and Wilson, M. Infectious disease pathology. *Clin. Infec. Dis.*, 2001; **32**: 1589-1601.