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Investigation of Phenotypic and Genotypic Characteristics of Isolation of Strains *Vagococcus salmoninarum* Cage Business Rainbow Trouts (*Oncorhynchus mykiss*) of Kahramanmaras Province (Turkey)

M. Özcan and Y. Baris
Department of Fisheries, Faculty of Agriculture, Kahramanmaras Sutcu Imam University, Kahramanmaras, Turkey
mikailozcan@ksu.edu.tr

Abstract: In this study, it was included to identify and investigate phenotypic and genotypic charecteristics the isolation of *Vagococcus salmoninarum* in rainbow trout farms the cage of Kahramanmaras Province (Turkey). For this purpose, farms twenty different trout between July 2014 and June 2013 were taken in samples the gut and kidney, spleen, liver, eye rainbow trout weighing 250-300 g. For isolation of *Vagococcus salmoninarum*; tryptic soy agar, nutrient broth and brain heart infusion agars were used. The pure strains obtained from these samples from trout farms were evaluated phenotypic characteristics applied with Biolog system (The biolog GENIII micro plate) connection and biochemical tests. Antibiogram sensitivity test was also applied, according to disk diffusion method. General primer were used to confirm 53 *Vagococcus salmoninarum* with PCR after determined, according to bacteria the phenotype and genotype characteristics. Subsequently, these strains were confirmed as *Vagococcus salmoninarum* with DNA. The RFLP (Restriction Fragment Length Polymorphism) the strain of confirmed PCR (Polymerase Chain Reaction) were measured of features gene by the technique. *Vagococcus salmoninarum* was made from sequence analysis one of the resulting sample and the analysis result was interpreted from data obtained. Sub-typing *Vagococcus salmoninarum*m with RFLP technique was performed successfully.

Key words: *Oncorhynchus mykiss*, phenotypic, genotypic, *Vagococcus salmoninarum*, Biolog GEN III, PCR, RFLP, sequence

INTRODUCTION

While, the primary pathogen bacterial agents (*Aeromonas* spp., *Pseudomonas* spp., *Flexibacter* spp., *Vibrio* spp., *Yersinia* spp., *Renibacterium* spp., *Streptococcus* spp.) are frequently observed in trout farming establishments in Turkey as well as the whole world, in has been reported in recent years that Gram positive cocci spread sporadically and endemically and that among these six different species (*Streptococcus parauberis*, *Streptococcus difficile*, *Streptococcus iniae*, *Vagococcus salmoninarum*, *Lactococcus piscium* and *Lactococcus garvieae*) are related with fish diseases (Hambali and Rukyani, 1992; Egidius, 1987; Larsen *et al*., 1988; Eldar *et al*., 1996, 1999; Dalsgaard and Madsen, 2000; Roberts, 2001; Timur and Korun, 2004; Avci *et al*., 2010; Austin and Austin, 1999).

Having been discovered for the first time in 1968 in adult rainbow trout in Oregon USA, *Vagococcus salmoninarum* (Wallbanks *et al*., 1990; Daly, 1999) was isolated, since, then from rainbow trout in Australia, France, Italy and Spain (Schmidtke and Carson, 1994; Michel *et al*., 1997; Ruiz-Zarzuela *et al*., 2005) and from *Salmo salar* and *Salmo trout* in Norway (Schmidtke and Carson, 1994). In Turkey, on the other hand, it was initially found in rainbow trout farms located in the Mediterranean Region (Didinen *et al*., 2011).

The disease caused by *Vagococcus salmoninarum* is referred to as cold water Gram positive cocci infection. In fishes the disease causes skin darkening, single or double eye exophthalmoses, belly ascites, liver paling, growth and blackening of spleen and kidneys, petechial haemorrhage of muscles, air bladder and stomach and yellowish purulent exudates at places. Having been initially observed as rare cases, the disease caused by *Vagococcus salmoninarum*m today causes significant losses in fish farming with its endemic or even epidemic progression. Particularly observed during seasons when water temperature is about 10-12°C and in the presence of other stress factors, the disease causes

Corresponding Author: Mikail Özcan, Department of Fisheries, Faculty of Agriculture, Kahramanmaras Sutcu Imam University, Kahramanmaras, Turkey, mikailozcan@ksu.edu.tr

mortality rates between 20 and 50% (Michel *et al*., 1997; Ruiz-Zarzuela *et al*., 2005; Austin and Austin, 2007). *Vagococcus salmoninarum's* effective catalyze that grow as Gram-positive short oval bacilli is negative; H_2S is positive and alpha haemolytic. Its VP reaction, on the other hand is negative (Miyazaki *et al*., 1984; Michel *et al*., 1997).

Phenotypic method reflects genetic characteristics and are generally specific. If a certain phenotypic characteristic is found rarely in a pathogenic agent, it may by itself give an idea concerning how the agent spreads. However, if agents having phenotypic characteristics that can be observed often are in question then sub-typology has to be carried out (Swaminathan and Matar, 1993; Olive and Bean, 1999).

In the determination of the phenotypic characteristics of bacteria usually microbiological tests are employed (Austin and Austin, 1987; Bernardet and Kerouault, 1989; Bernardet *et al*., 1996). Biolog GEN III MicroPlate (Biolog 21124 Cabot Blvd. Hayward, CA 94545) is a standard auto-identification system where 94 biochemical tests are used in combination in order to identify Gram-negative and Gram-positive bacteria at species-level (Bochner, 1989).

Polymerase Chain Reactionn (PCR) is a method leading the reproduction of targeted nucleic acid as biochemically in order to determine genetic material of the required factors in a short time (Saiki *et al*., 1988; Mullis, 1990; Bej *et al*., 1991; Taylor, 1993).

With the development of PCR, RFLP analysis of DNA's parts amplified via. PCR has become a common method in genetic studies (Hall and Nawrocki, 1995; O'Connell *et al*., 1995; Hansen and Loeschcke, 1996). Another method that has been used widely in the area of aquacultural resources is PCR-RFLP applications. By means of this method, species can be distinguished by using their genetic characteristics.

Sensitivity to antibacterial preparations can be determined through several methods. Bacteriostatic and bactericide activities of preparations are generally assessed with routine tests carried out in laboratories (Baker *et al*., 1991; Jorgensen, 1997; Lalitha, 2004).

In the present postgraduate thesis, culture method that is used in the diagnosis of bacterial fish diseases was employed as well as the Biolog system for determining other phenotypic characteristics and RFLP technique for molecular typing. *Vagococcosis* that is a serious bacterial disease in rainbow trout farming was examined and the phenotypic and genotypic characteristics of the agent were determined. Also, sensitivity levels of the isolates to antibiotics were determined by conducting antibiogram tests. In this way morphological, biochemical, physiological and genetic differences of the agent isolated from the target area were set forth as regards to reference strains.

MATERIALS AND METHODS

Fish: The 20 trout farming establishments that are subject to this research are located in Kahramanmaras Province (Turkey). Between the dates of June 2013 and July 2014, samples were collected from the eyes, livers, spleens, kidneys and intestines of rainbow trout weighing between 250 and 300 g in 20 establishments.

Reference strain: For the purposes of determining the phenotypic characteristics of *Vagococcus salmoninarum* suspected isolates, detecting a possible contamination in any phase of the application of PCR technique, applying the appropriate DNA extraction method, optimizing RFLP technique and carrying out genetic typing, *Vagococcus salmoninarum* NCIMB 13133 reference strain was obtained from ATCC laboratory.

Enzyme and kit: BoxI and BseMI enzymes were used for RFLP technique. In the sequence phase ExoSAP-IT, BigDye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems) and Sephadex were utilized.

Microbial isolation: For the isolation of *Vagococcus salmoninarum* from trout establishments, the samples collected were planted on Tryptic Soy Agar (TSA) and Brain Heart Infusion Agar (BHIA) by conducting abdominal incision from liver, spleen, kidney and intestines under aseptic conditions. The samples were left in the incubator for 48 h at 18°C. Pure cultures were obtained from the colonies that completed reproduction.

Biochemical and Phenotypic characterization: For identification purposes colony morphology, gram staining and cell morphology, movement examination, potassium hydroxide (KOH), gram reaction, catalase, oxidase, Oxidation/Fermentation (O/F), Methyl Red (MR), Voges Proskauer (VP) and Indol tests were carried out. In addition for the purposes of validating biochemical tests and determining other phenotypic characteristics the standard auto-identification system Biolog GEN III MicroPlate where 94 biochemical tests are used in combination in order to identify Gram-negative and Gram-positive bacteria at species-level was utilized (Bochner, 1989). Antibacterial sensitivity tests were carried out in line with disc diffusion method (Bauer *et al*., 1966; Bowser and Plumb, 1983; Koneman *et al*., 1997; Furones, 2001).

DNA extraction: Chromosomal DNA was obtained from *Vagococcus salmoninarum* strain with chromosomal DNA isolation kit in order to be used in the reproduction of the genes to be cloned.

Polymerase Chain Reaction (PCR): PCR operation was carried out at a total of 40 μL volume with the use of DNA polymerase enzyme. Also, in colony PCR studies our own laboratory production DNA polymerase was used. For the reaction the total volume (40 μL) was completed with 1 μL (20 pikomol) forward primer (356F: ACWCCTACGGGWGGCWGC), 1 μL (20 pikomol) reverse primer 1064R: AYCTCACGRCACGAGCTGAC), 1 μL dNTP (1 mM), 4 μL buffer $(NH4)_2SO_4$ (10X), 1.6 μL $MgCl_2$ DNA polymerase (5 u/μL) [25 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 1 mM DTT and 50% (v/v) glycerol], 1 μL template DNA (~400 ng/mL) and 29.4 μL dH_2O. In PCR operation 94°C DNA denaturation, variable adhesion, 72°C elongation temperature were set and PCR was carried out in 30 cycles. In colony PCR applications, before the 1st PCR cycle a pre-denaturation stage of 5 min at 95°C was implemented. PCR products were separated by electrophoresis in 2% (w/v) agarose gels and visualized by ethidium bromide staining. A 100 bp DNA ladder (MBI Fermentas) molecular mass marker was used to evaluate the size of bands.

Nucleotide sequencing: DNA sequencing was carried out by means of ABI3 130xl gene sequence equipment. Sequencing covers ExoSAP purification, Cycle PCR and Sephadex purification phases. ExoSAP purification consists of adding 2 μL ExoSAP-IT enzyme to 5 μL PCR product and 15 min of inactivation at 80°C after 30 incubation period at 37°C. In the Cycle PCR stage BigDye Terminator V3.1 was used in line with Cycle Sequencing Kit (Applied Biosystems) protocol. Sephadex purification was performed with the use of Sephadex G-50. Sequencing of clone fragments that were obtained with the use of CloneJET Kit was carried out by means of pJET 1.2 forward and pJET 1.2 reverse primers. In the sequencing made on pGEM-T vector, M13 primer was used. DNA sequences of PCR products were made by using forward and reverse primers separately and the sequences were analyzed with ChromasPro and Clone Manager 9 Software.

RFLP (Restriction Fragment Length Polymorphism): Two different enzymes were used in the research. While BoxI (PshAI) is incubated at 37°C BseMI is incubated at 55°C. The 10 mL mixture prepared for each enzyme was left for incubation in water baths at 37 and 55°C and then inactivated at 80°C. Consequently, they were placed in succession with their positive samples in the 1% agarose gel. The process was run at 120 V and 60 min. Samples were observed under UV light and photographed.

RESULTS AND DISCUSSION

From the anamnesis taken from the fish farm establishments in Kahramanmaras Province it was determined that particularly among the fishes weighing between 250 and 300 g the mortality rate in 1 week of period is 60% and that the fishes are mostly idle and lacking appetite. It was determined that the waters were between 12-16°C, 7.01-7.5 pH and 7.5-9 mg/L O_2. Skin darkening, single or double eye exophthalmoses, belly ascites, liver paling, growth and blackening of spleen and kidneys, petechial haemorrhage of muscles, air bladder and stomach and yellowish purulent exudates at places were observed in the diseased fishes.

From the pure cultures of 48 isolated Gram positive cocci shaped *Vagococcus salmoninarum* strains their phenotypic and biochemical characteristics were determined Table 1. Also, in order to validate biochemical tests and determine other phenotypic characteristics Biolog system equipment was used Table 2. After the isolation and identification of *Vagococcus salmoninarum* 5 different antibiogram disc were used for antibiogram sensitivity test (Table 3). PCR pictures taken with the use of the pure cultures of 53 isolated Gram positive cocci shaped *Vagococcus salmoninarum* strains are presented in Fig. 1.

Table 1: Morphological and biochemical characteristics of 53 *Vagococcus salmoninarum* isolated from rainbow trout

Phenotypic and biochemical features	*Vagococcus salmoninarum* (n:53)
Colony color	White
Gram coloring	+
Shape	Coccobacillus
Oxidase	-
Catalase	-
Movement	-
H_2S	-
Methyl red	-
Voges proskauer	-
Indole	-
Urease formation	-
Oxidation/fermentation	F
MacConkey agar	-
Mueller-Hinton agar	+
Reproduction at 0°C	-
Reproduction at 5°C	+
Reproduction at 15°C	+
Reproduction at 20°C	+
Reproduction at 25°C	+
Reproduction at 30°C	+
Reproduction at 37°C	+
Reproduction in 0.0% NaCl	+
Reproduction in 0.5% NaCl	+
Reproduction in 1.0% NaCl	+
Reproduction in 2.0% NaCl	+
Reproduction in 6.5% NaCl	-

Table 2: Other phenotypic characteristics with Biolog system of 53 *Vagococcus salmoninarum* isolated from rainbow trout

Biochemical criteria	Isolate reaction (n: 53)	Biochemical criteria	Isolate reaction (n: 53)
pH 5	+/-	NaCl (8%)	-
pH 6	+	NaCl (4%)	+/-
Positive control	+	NaCl (1%)	+
Stachyose	-	N-Acetyl neuraminic acid	-
D-Turanose	-	N-Acetyl-D-Galactosamine	+/-
Sucrose	+	N-Acetyl-β-D-Mannosa-mine	+/-
Gentiobiose	+/-	N-Acetyl-D-Glucosamine	+
D-Cellobiose	+/-	D-Salicin	+/-
D-Trehalose	+	β-Methyl-D-Glucoside	+/-
D-Maltose	+/-	D-Melibiose	-
Dextrin	+	α-D-Lactose	-
Negative control	-	D-Raffinose	-
D-Serine	-	Minocycline	+
Fusidic acid	-	Rifamycin SV	+
Sodium lactate (1%)	+	Troleando-mycin	-
I Nosine	-	D-Serine	-
L-Rhamnose	-	D-Aspartic acid	-
L-Fucose	-	D-Fructose-6-Phosphate	-
D-Fucose	-	D-Glucose-6-Phosphate	-
3-Methyl glucose	-	Glycerol	-
D-Galactose	-	Myo-ynositol	-
D-Fructose	+	D-Arabitol	-
D-Mannose	+	D-Mannitol	-
a-D-Glucose	+	D-Sorbitol	-
Niaproof 4	+/-	Tetrazolium blue	+/-
Guanidine HCl	+/-	Tetrazolium violet	+
Lincomycin	+/-	Vanco-mycin	+/-
L-Serine	-	D-Saccharic acid	-
L-Pyroglutamic acid	-	Quinic acid	-
L-Histidine	-	Mucic acid	-
L-Glutamic acid	-	Glucoronamide	-
L-Aspartic acid	-	D-Glucoronic acid	-
L-Arginine	-	D-Gluconic acid	-
L-Alanine	-	L-Galactonic acid lactone	-
Glycyl-L-proline	-	D-Galacturonic acid	-
Gelatin	-	Pectin	+/-
Potassium tellurite	+	Sodium bromate	+
Lithium chloride	+	Sodium butyrate	+
Nalidixic acid	+	Aztreonam	+
Bromo-succinic acid	-	Formic acid	-
L-Malic acid	-	Acetic acid	-
D-Malic acid	-	Propionic acid	-
a-Keto-glutaric acid	-	Acetoacetic acid	+/-
Citric acid	-	αKeto-Butyric acid	+/-
L-Lactic acid	-	β-Hydroxy-D,L-Butyric acid	-
D-Lactic acid methyl ester	-	α-Hydroxybutyric acid	-
Methyl pyruvate	-	γ-Amino-Butryric acid	-
p-Hydroxy-phenylacetic acid	-	Tween 40	-

(a)

Fig. 1: Image of PCR samples 53

Gel images of the RFLP results obtained by using pure cultures of *Vagococcus salmoninarum* are as follows. Band images obtained from the cut made with BoxI enzyme are presented in Fig. 2 while those obtained from the cut made with BseMI enzyme are presented in Fig. 3.

Fig. 2: a and b) Band images of examples 1-15 obtained cutting result made with BoxI enzyme

Table 3: Antibiogram test result of 53 *Vagococcus salmoninarum* isolated from rainbow trout

Antibiotics	Sensitivity (mm)
Erythromycin (15 μg)	S (25)
Streptomycin (10 μg)	R (0)
Enrofloxacin (5 μg)	S (22)
Sulfafurazole (10 μg)	R (0)
Florfenicol (30 μg)	S (30)

Sequence analysis was made, for sample No. 51, among *Vagococcus salmoninarum* strains. While the nucleotide sequence obtained from the NCBI of *Vagococcus salmoninarum*m is presented in the nucleotide sequence obtained from the sample No. 51. These samples were aligned with Clone Manager 9 Software and the result (Table 4).

Within the scope of this research *Vagococcus salmoninarum* was isolated and identified by means of the conventional culture method from rainbow trout that were suspected to be diseased in 20 different trout farming establishments in Kahramanmaras Province. In addition with the use of Biolog system equipment biochemical tests were verified and other phenotypic characteristics were determined.

Fig. 3: a and b) Band images of examples 1-15 obtained cutting result made with BseMI enzyme

Within a period of 1 week, the disease exhibited a mortality rate of 60% in waters at 12-16°C temperature. This rate was found to be higher than those reported in previous studies (Michel *et al*., 1997; Ruiz-Zarzuela *et al*., 2005; Didinen *et al*., 2011). This difference is related with environmental conditions, stress factors and intense stocking. Skin darkening, single or double eye exophthalmoses, belly ascites, liver paling, growth and blackening of spleen and kidneys, petechial haemorrhage of muscles, air bladder and stomach and yellowish purulent exudates at places were observed in the diseased fishes. These clinical findings are in parallel with the findings obtained in studies conducted in other countries (Michel *et al*., 1997; Ruiz-Zarzuela *et al*., 2005; Didinen *et al*., 2011).

While in the studies conducted by Michel *et al*. (1997) and Ruiz-Zarzuela *et al*. (2005) *vagococcus* was found in waters of 10-12°C temperature, Didinen *et al*. (2011) reported *vagococcus* in waters at 12.6°C. In the present study the disease was found at 12-16°C. Bacterial isolate's identification test results (Table 1 and 2) obtained within the scope of this research were found out

Table 4: The 16S sequence alignment of example 51

Examples	Values
Reference	362gcagcngtagggaatcttcggcaatggacgaaagtctnaccgagcaacgccgcgtgagtg
Example 51	669 .....a.....-..c.....g.-.....c.....
Reference	422 aagaaggttttcggatcgtaaaactctgttgttagagaagaacaagtgggagagtaactg
Example 51	611 .....a.....gat.....
Reference	482 ttcccaccttgacggtatctaaccagaaagccacggctaactacgtgccagcagccgcgg
Example 51	551 c..atc..c.....t.....
Reference	542 tnatacgtaggtggcnagcgttgtccggatttnttgggcgtnaagcgagcgcaggtggtt
Example 51	491 .a.....a.....a.....a.....c....
Reference	602 ctttaagtctnatgtgaaagcccccggctcnaccgggcagggtcattggaaactggggna
Example 51	431 .....g.....a.....g.....a.a.
Reference	662 cttgagtgcagaagaggagagtggaattccatgtgtagcggtgaaatgcgtagatatatg
Example 51	371 .....c.....g..
Reference	722gaggaacaccagtggcgaaggcgactctctngtctgtaactgacactnaggctcgnaagc
Example 51	311 .....g.....g..g.....a....
Reference	782gtggggagcaaacaggattagataccctngtngtccacgccgtaaacgatgagtgctnag
Example 51	251 .....g..a.....a..
Reference	842 tgttggagggtttccgccccttcagtgctgcagtnnacgcattaagcactccgcctnggga
Example 51	191 .....cta.....g....
Reference	902 gtacggtcgcaagactnaaactcaaaggaattgacgggga-ccgcacaagcggtggagc
Example 51	131 .....c.....g..g.....c.....
Reference	961 tgtggtttaattcgaagnaacgcgaagaaccttaccaggtcttgacatcccttgaccact
Example 5	171 .....c.....t.....
Reference	1021 cgagagat
Example 51	11 .t.....

to be in line with the previously obtained knowledge on *V. salmoninarum* (Michel *et al*., 1997; Ruiz-Zarzuela *et al*., 2005; Didinen *et al*., 2011). According to the bands obtained from the conducted PCR studies all samples exhibited positive bands images as it was the case in previously conducted studies. However, in the present research general primer was used in PCR studies. As the next step of the research primer design should be carried out and PCR studies should be repeated. Among the bands obtained with RFLP technique no assessable positive band could be found. In following studies typing should be repeated with the use of different enzymes.

Among the strains of *Vagococcus salmoninarum* sample No. 51 was subjected to sequence analysis. However, due to the facts that only one sample was sequenced and only one-directional successful reading could be made the result may not be healthy and thus it is suggested for following studies that all samples should be subjected to sequence analysis and the presence of the species should be asserted only after that. Within the scope of the present study *Vagococcus salmoninarum* was isolated and identified by means of conventional culture method from disease-suspected trout weighing between 250-300 g in 20 different trout farming establishments in Kahramanmaras Province.

CONCLUSION

Concerning the Biolog system (The Biolog GEN III MicroPlate), 94 phenotypic tests consisting of *V. salmoninarum's* 71 different carbon source usage and 23 chemical sensitivity tests were conducted successfully. The strains verified via. PCR were examined in terms of genetic characteristics with RFLP technique. One of the obtained *Vagococcus salmoninarum* samples was subjected to sequence analysis and the data collected from the analysis were evaluated. As a result *Vagococcus salmoninarum's* phenotypic and genotypic characteristics were set forth.

In culture fishery as a result of fishes being in close contact with each other, the rapid contamination of the water and thus, the quality of water deteriorating beyond optimal values cold water Gram positive cocci infection occurs and spreads rather easily. Due to this reason the necessary protective measures should be taken in a timely manner and changing living conditions should be rectified. Stress causes fishe's immunity system to shut down and therefore, leave the fish susceptible for *Vagococcus salmoninarum*. Due to this reason in order to ensure that the fishes are not subjected to stress culture conditions should be set at optimum, high water quality should be maintained, proper feeds should be used, use of unnecessary medicine and chemicals should be avoided and noise levels should be minimized.

Using antibiotics is a commonly followed method for fighting cold water Gram positive cocci infection. However, since, antibiotics gradually cause water pollution, residues in fish, growth of antibiotic-resistant microorganisms and adverse effects on aquatic environment and human health it should be tried to abstain from this method. The most effective method for fighting diseases is to take the necessary measures to avoid the disease at the first place, rather than trying to stop it after it occurs. Therefore, the most effective method in fighting epidemic fish diseases is vaccination.

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