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Identification and Investigation of Phenotypic and Genotypic Characteristics of Flavobacterium Psychrophılum ın Fry Raınbow Trouts (Oncorhynchus Mykıss) in Some Trout

Mikail ÖZCAN[1], Mustafa SARIEYYÜPOĞLU[2]

[1]Department of Fisheries, Faculty of Agricultural, University of KSU, Kahramanmaras, TURKEY
[2]Firat University, Fisheries Faculty, Department of Fish Diseases, Elazig, TURKEY

Abstract: In this study, it was aimed to identify and investigate phenotypic and genotypic charecteristics of *Flavobacterium psychrophilum* in four rainbow trout farms in Keban district of Elazığ province. Two different primers were used to confirm 160 *Flavobacterium psychrophilum* with PCR after determined according to the phenotype and genotype characteristics. Subsequently, these strains were confirmed as *Flavobacterium psychrophilum* with DNA. Gene typing was performed with PFGE technique on strains confirmed with PCR. As a result of this, it was decided that strains obtained from four different fish farms wasclose relative with reference *Flavobacterium psychrophilum* NCIMB 1947^T.

Keywords: *Flavobacterium psychrophilum*, Rainbow trout, *Oncorhynchus mykiss*, Polymerase Chain Reaction (PCR), Pulsed Field Gel Electrophoresis (PFGE)

Introduction

Bacterial fish disease in culture fishing is one of the most important factors that cause economic losses in businesses. Severity of the disease depends on the type and age of the fish. But many infections in fish can be successfully treated. However, the negativity of environmental conditions makes it difficult to protect fish from diseases and to take control measures and even makes it impossible (Austin and Austin,1987; Buckley et al., 1998).

Psikrofiloz (Bacterial cold water disease, peduncle disease, Rainbow Trout Fry Syndrome -RTFS) is a septicaemic characterized, acute progressive, epidemic and lethal infection which is caused by *F. psychrophilum* and which occurs especially in hatcheries where the water temperature falls below 10C^o. For this reason, it causes economical losses for enterprises. Psikrofilozis was detected for the first time in Turkey in fry rainbow trout in 1993 and it caused serious economical losses in fish production plants after it had spread in different geographical regions in the subsequent years. So far, in our country, this disease has been commonly seen in February-June and has become an important bacterial disease of fry rainbow trout (Bernardet and Kerоualt, 1989; Austin, 1992; Lumsden *et al.*, 1996; Diler *et al.*, 2003; İspir *et al.*, 2004; Arda *et al.*, 2005).

F. psychrophilum resides on skin, mucosa, fins, gills, operculum, ovarian fluid and natural flora of the digestive system and causes disease as well (Diler *et al.*, 2000; Nematollahi *et al.*, 2003; Arda *et al.*, 2005; Kubilay *et al.*, 2009a; Kubilay *et al.*, 2009b). In addition, it has been reported that the factor may infect the eggs of sick spawner fish and then the juvenile fish (Balta, 1997; Diler *et al.*, 2003). As the environmental factors change, virulence of the factor increases too and causes infection. Because the factor infects via contact and water spreading quickly, it causes excessive deaths in rainbow trout farming enterprises and eventually significant economic losses in those enterprises (Cipriano and Holt, 2005).

Microbiological tests are often used to determine the phenotypic characteristics of *Favobacterium psychrophilum* (Arda, 2000; Austin and Austin, 1987; Bernardet and Kerouault, 1989; Bernardet *et al.*, 1996).

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).

Pulsed Field Gel Electrophoresis (PFGE) is used as a reference in genotypical identification because it has very high separation power and repeatable results and because it is easy to interpret. This method is based

Mikail ÖZCAN (Correspondence)
mikailozcan@ksu.edu.tr
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on the basis of cutting with restriction enzyme for the determination of genetic profile without deterioration in structural integrity of the chromosome isolated from agarose-embedded bacteria (Derbentli, 2002; Domig *et al.*, 2003; Van Belkum *et al.*, 2007).

There has not been an extensive research on phenotypic and genotypic characters of *F. psychrophilum* which is an important factor of Psikofiloz which is one of the important fish diseases in our country. In this study, it is aimed to investigate phenotypic and genotypic characteristics of this factor and to determine *Flavobacterium psychrophilum* bacteria in fry rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) in the hatcheries of some rainbow trout farming enterprises in Keban district of Elazığ province.

Materials and Methods

Bacterial isolates

A total number of 4024 *F. psychrophilum* isolates were examined in this study (Table 1). The Turkey collection contained 160 isolates, isolated from diseased salmonids between December 2008 and July 2009. The type strain of *F. psychrophilum* (NCIMB 1947^T) was included in all analyses All isolates were stored at -80 °C until used.

Table1. I, II, III, IV of farm isolated samples taken from the number and percentage of *Flavobacterium psychrophilum.*

SAMPLE TAKEN PLACE		EXAMPLE FROM FARMS						
		I. farm	II. farm	III. farm	IV. farm	Total Number of Materials	Number of *Flavobacterium psychrophilum*	Percentage Rate
Eggs		40	40	50	45	175	18	10.28
Ovarian Liquid (2 ml)		40	50	50	45	185	13	7.02
Sperm (2 ml)		50	55	40	55	200	0	0
Eggs Washed With Povidin/Iodin Solution		40	40	50	55	185	6	3.24
Fertilized Eggs		50	55	60	40	205	14	6.82
Eyed Eggs		60	60	60	60	240	14	7.77
Larvae with Egg Sac		100	150	120	140	510	13	2.54
Fry Under 5 g		100	100	100	100	400	15	3.75
Ealthy Fry over 5 g	Spleen	50	50	50	50	200	8	4
	Liver	50	50	50	50	200	5	2.5
	Kidney	50	50	50	50	200	4	2
	Intestine	50	50	50	50	200	1	0.5
Healthy of newly dead and Suspicious from disease fry over 5 g	Spleen	50	50	50	50	200	17	8.5
	Liver	50	50	50	50	200	10	5
	Kidney	50	50	50	50	200	10	5
	Intestine	50	50	50	50	200	6	3
Food		30	30	30	30	120	0	0
Water		64	60	40	40	204	6	2.94

Culture media

For grown of *Flavobacterium psychrophilum*; Tryptone Yeast Extract Salt Agar (TYES-A), (Holt, 1987; Michel et al., 1999) (0.4% tryptone, 0.04% yeast extract, 0.05% calcium chloride, 0.05% magnesium sulphate, 1.1% agar pH 7.2) and Enriched Anacker and Ordal + Fetal Bovine Serum+ Agar (EAO+FBS+A) (0.5% tryptone, 0.05% yeast extract, 0.05% beef extract, 0.02% sodium acetate, 0.02% calcium chloride, 0.05% magnesium sulfate, and 5% fetal bovine serum, 1% agar) (Starliper vd., 2007), were used.

Biochemical and physiological characterization

Flavobacterium psychrophilum isolates were identifed and characterized using the following tests, as described by Bernardet (1989): catalase, cytochrome oxidase, absorption of Congo red, presence or absence of fexirubin-type pigment, production of acid from glucose, fructose, galactose, mannose and glycerol, production of amylase and caseinase, tyrosine hydrolysis, reduction of nitrate and production of hydrogen sulphide using commercial lead acetate paper strips.

Gram staining was carried out according to Collins & Lyne (1976). Gliding motility was tested by direct microscopy of agar cultures (24±48 h). A coverslip was placed on the agar at the edge of the bacterial growth. Gliding motility was assessed by phase-contrast microscopic examination of a fresh TYES broth culture and by the hanging drop technique (Bernardet et al. 2002). Anaerobic growth of the isolates was tested on agar plates incubated in an anaerobic jar for 7 days. Growth of the isolates was tested on Tryptone Yeast Extract Salt Agar (TYES-A), (Holt, 1987; Michel et al., 1999) and Enriched Anacker and Ordal + Fetal Bovine Serum+ Agar (EAO+FBS+A)) (Starliper vd., 2007) for 7 days. Growth at different temperatures was determined by inoculating bacterial cells in early log-phase into TYES and incubating the cultures at 5, 20 and 25 °C for 7 days. The growth was evaluated by eye. Production of gelatinase and elastinase was tested using AO agar supplemented with 1% gelatine and 0.05% elastine, respectively. The plates were streak inoculated and observed for subsequent clearance around the colonies during 14 days. Esculin hydrolysis was tested in TYES containing 0.1% esculin and 0.05% ferric citrate. The tubes were incubated for 7 days and a dark brown or black colour was considered as a positive result. Lysis of cells was tested on TYES with added washed and autoclaved yeast cells (bakers yeast). Clearing around colonies after 14 days of incubation indicated lysis of the yeast cells. All tests were carried out at 15 °C unless otherwise indicated.

DNA Extraction

For the DNA extraction from cultures; a few represen-tative colonies from pure cultures were transferred into an Eppendorf tube containing 300 µl distilled water. The bacterial suspension was treated with 300 µl TNES buffer (20 mM Tris pH 8.0, 150 mM NaCl, 10 mM EDTA, 0.2% SDS) and 5 µl proteinase K (200 µg ml–1), and was kept at 37°C for 2 h. Following 10 min of boiling, an equal amount of phenol: chloroform:isoamylalchol (25:24:1) was added to the suspen-sion. The suspension was shaken vigorously by hand for 5 min and then centrifuged at 13.000 g for 10 min. The upperphase was carefully transferred into another Eppen-dorf tubeand 3 M sodium acetate (0.1 volumes) and 95% ethanol (2.5volumes) were added to the suspension, which was leftat –20°C overnight to precipitate the DNA. The pellet, ob-tained following the centrifugation at high speed for 10min was washed twice with 90 and 70% ethanol respectively, each step was followed by 5 min centrifugation. Finally, the pellet was dried and resuspended in 50 µl distilled water.

Polymerase Chain Reaction (PCR)

For the PCR analysis of *F. psychrophilum* suspected isolates, the reaction mixture was prepared in a total volume of 50 µl, consisting of 5 µl DNA, 10x PCR buffer (750 mM Tris/HCl, 200mM (NH4)2SO4, 0.1% Tween 20), 5 µl 25 mM MgCl2, 250 µMeach of deoxynucleoside triphosphates, 1.25 U *Taq* DNApolymerase (MBI Fermentas) and 20 pmol each of primerpair derived from 16S rDNA gene of *F. psychrophilum* PSY1 190–206 (5'-GTT GGC ATC AAC ACA CT-3') ve PSY2 1278–1262 (5'-CGATCC TAC TTG CGT AG-3') 50 pmol and 5µl target DNA (Suzuki vd., 2008). The PCR analysiswas performed in a thermal cycler with an initial denatura-tion step at 94°C for 5 min, followed by 35 cycles at 94°C for 30 second, 51°C for 90 second and 72°C for 2 min, then, a last step of extension at 72°C for 5 min. 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.

A reference strain of *Flavobacterium psychrophilum* NCIMB 1947^T which was obtained from Centro de Biotecnología Animal Laboratory in Spain was used in order to determine the most suitable DNA extraction procedure and to optimize PCR.

Pulsed-field gel electrophoresis (PFGE)

The 160 isolates of *F. psychrophilum* were analysed by PFGE, which was performed as described previously (Barrett et al. 1994; Izumiya et al. 1997) with minor modifications. Single colonies of *F. Psychrophilum* were inoculated onto Enriched Anacker and Ordal + Fetal Bovine Serum+ Agar

(EAO+FBS+A)) (Starliper vd., 2007) and incubated at 15 °C for 72 h. *Flavobacterium psychrophilum* grown on EAO+FBS+A was suspended in 3 ml of sterilized distilled water (Arai, *et al.*, 2007). Bacterial cells from 1ml aliquots were harvested, washed twice with cell suspension buffer (CSB: 100 mM Tris,100 mM EDTA, pH 8.0) and resuspended in the same buffer until an optical density (OD) of 1.2 was obtained. The OD was measured at 610 nm. Bacterial suspensions were incubated with 20 µl Proteinase K (20 mg/ml) 37 °C for 15 min, mixed with an equal volume of 1% chromosomal grade agarose (Seakem GTG agarose, Cambrex Corporation) and immediately loaded into a disposable plug mould (Bio-Rad Laboratories) and allowed to solidify at room temperature. Bacterial cells in each plug were then lysed in lysis buffer (50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl) and 25 µl proteinase K (20 mg/ml) for 2–3 h at 56 °C with shaking. Prior to digestion with restriction enzyme, the plugs were washed twice for 30 min with distilled water at 50 °C and four times for 15 min with Tris–EDTA buffer (TE: 10 mm Tris–HCl [pH 8], 0.1 mm EDTA). The plugs were stored in TE at 4 °C until use. *XhoI* and *BInI* of restriction endonucleases were used in the study. The DNA in the plugs were cut in half, the individual pieces were incubated in 200 lL of buffer for each enzyme at 4 °C for 30 min. The DNAs in each plug were digested with 50 units of each restriction endonuclease at 37 °C for 4 h. PFGE was performed using a CHEF DR II apparatus (Bio-Rad Laboratories), with 1% agarose gel in 0.5x TBE (Tris-borate-EDTA) buffer at 6 V cm)1 at 12 °C. For separation of the whole genome, a linearly ramped switching time from 1 to 12 s was applied for 18.5 h. After PFGE, the gels were stained with ethidium bromide (10mg/ml) and viewed and photographed under UV light. Digital images were stored electronically as TIFF files. After visual inspection of the banding patterns obtained by PFGE, computer analyses were carried out using BioD 1++ (infinity capt software, Vilber Lourmat, Fransa). A dendrogram was constructed using the unweighted pair-group method with arithmetic averages (UPGMA), with the Dice coefficient and a tolerance of 1%.

Results

Physiological and biochemical reactions

The *F. psychrophilum* isolates were rather homogeneous in their physiological and biochemical reactions and were identical to those of the type strain NCIMB 1947^T. Positive reactions were obtained for catalase (Weak +), cytochrome oxidase and presence of fexirubin-type pigment, Oxidatio, Hemolysis, gelatine, tyrosine, Tween 20, Tween 80 and casein hydrolysis, growth at 5 and 20 °C and growth on Plate Count Agar and Mueller-Hinton Agar. Negative reactions occurred for Gram stain, Motion, Congo red adsorption, Indole, Metil Red, Simmons sitrate, Üreaz, H^2S, ONPG, OD, Voges Proskauer, Oxidase Nitrate, starch Hydrolysis esculin hydrolysis, acid production from carbohydrates, 0°C growth, 30°C growth, 37°C growth and anaerobic growth. Variable reactions were seen for growth at 25 °C (16 of 160), growth in TSB (32 of 160), growth in TSA(32 of 160) and Fermentation (48 of 160).

Specificity of the primers

The primers PSY1190–206 and PSY2 1278–1262 generated a fragment of identical size (1089 bp) from all tested strains of *F. psychrophilum* including the type strain NCIMB 1947^T (Fig. 1).

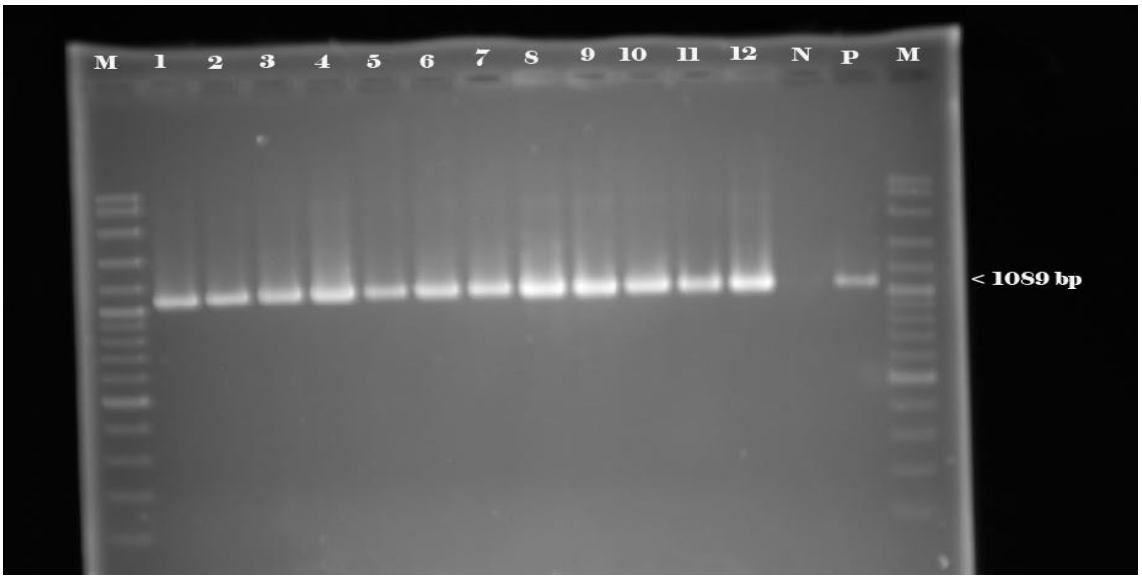

Figure 2. The agarose gel that shows 1089 bp length bands that belongs to *F. psychrophilum* type formed as a result of PCR analysis using PSY1 190–206, PSY2 1278–1262 primer pairs of DNA samples; 1-12: Samples of *F.*

psychrophilum isolated from enterprises; M: 100 bp molecular marker; N: negative control; P: Positive control reference strain.

PFGE analysis

Isolates that were identified as *F. psychrophilum* from egg for Molecular sub-Identification of *Flavobacterium psychrophilum* with Pulsed Gel Electrophoresis, ovarium fluid, egg disinfectated with providine/iodine, fertilized eggs, observed eggs, marsupial fry, fry ligther than 5 grams, spleen of fry heavier than 5 grams, kidney of fry heavier than 5 grams, liver of fry heavier than 5 grams, intestine of fry heavier than 5 grams were subjected to PFGE analysis with *Xhol* enzyme. As a result of PFGE analysis, the same band profile was detected in all the isolates obtained from four different enterprises (Figure 3).

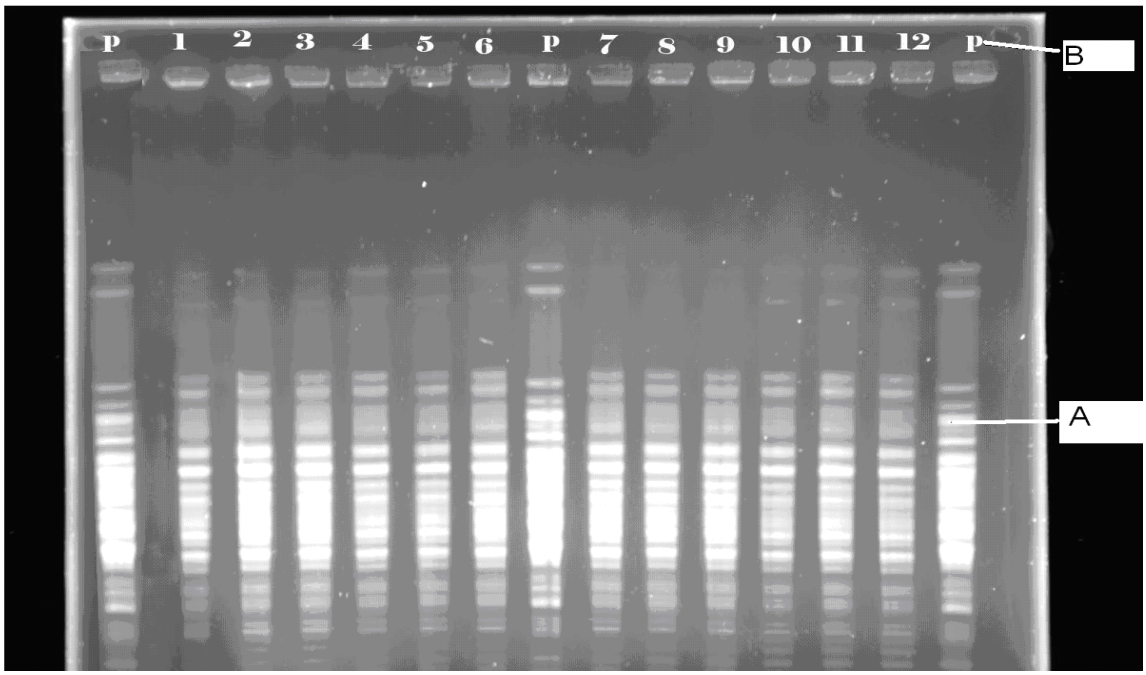

Figure 3. The bands on agarose gel as a results of cutting Choromosal DNA of *F. psychrophilum* with XhoI enzyme (A); P: *F. psychrophilum* NCIMB 1947^{T} reference strain (B); 1: egg; 2: Ovarian fluid; 3: Egg disenfactated with Povidine/ iodine; 4: fertilized egg; 5: observed egg; 6: Marsupial fry; 7:fry rainbow trout lighter than 5 grams; 8: spleen of fry rainbow trout heavier than 5 grams; 9: Spleen of fry rainbow trout heavier than 5 grams; 10: liver of fry rainbow trout heavier than 5 grams; 11: kidney of fry rainbow trout heavier than 5 grams; 12: intestine of fry rainbow trout heavier than 5 grams.

DNA isolation from agarose molds cotaining 160 pieces of *F. psychrophilum* confirmed by PCR and 3 pieces of *F. psychrophilum* NCIMB 1947^{T} reference strain was performed. 12 samples were chosen among these agarose mold DNA. Along with these samples, agarose molds containing 3 reference strain (P) were subjected to PFGE analysis with *Blnl* enzyme (Figure 4). At the end of PFGE analysis, the same band profile was detected in all of the isolate obtained from four different enterprises.

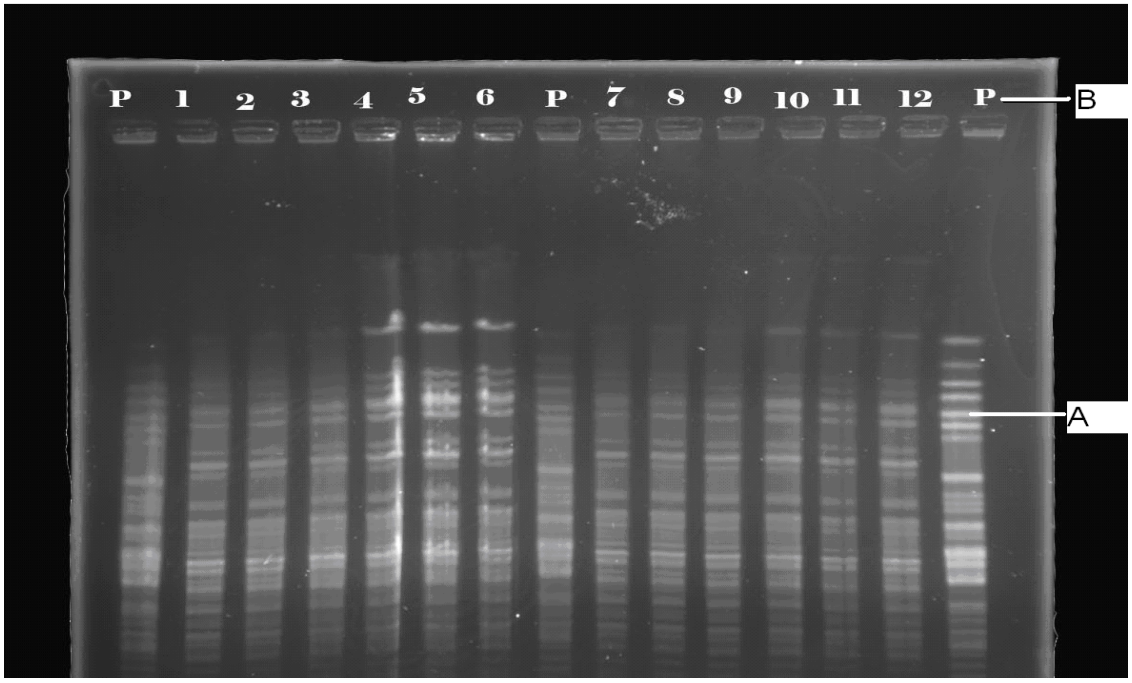

Figure 4. The bands on agarose gel as a results of cutting Choromosal DNA of *F. psychrophilum* with Blnl enzyme (A); P: psychrophilum NCIMB 1947^T reference strain (B); 1: egg; 2: Ovarian fluid; 3: Egg disenfactated with Povidine/ iodine; 4: fertilized egg; 5: observed egg; 6: Marsupial fry; 7:fry rainbow trout lighter than 5 grams; 8: spleen of fry rainbow trout heavier than 5 grams; 9: Spleen of fry rainbow trout heavier than 5 grams; 10: liver of fry rainbow trout heavier than 5 grams; 11: Kidney of fry rainbow trout heavier than 5 grams; 12: intestine of fry rainbow trout heavier than 5 grams.

When the profile analysis of the bands was performed using gel comparison software system, it was revealed that there was an 80% relationship between *F. psychrophilum* strain and reference strains (Figure 5). Accordingly, it was observed that there was a close relation between *F. psychrophilum* reference strain and isolates obtained from the enterprises. The reference strain profile of *F. psychrophilum* was identified as Type A and the isolates taken from the enterprises were identified as Type A_1 as they had the same profile.

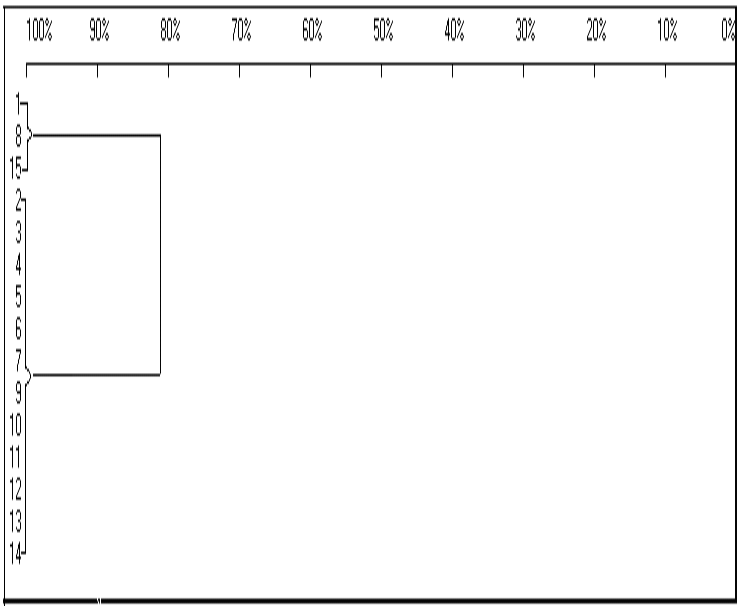

Figure 5. PFGE profile of *F. psychrophilum* reference strain and *F. psychrophilum* obtained from the enterprises. **1,8,15:** *F. psychrophilum* NCIMB 1947^T reference strain; **2,3,4,5,6,7,9,10,11,12,13,14:** *F. psychrophilum*

isolated from the enterprises.

Discussion and Conclusions

In this research, primarily the isolation and identification of *F. psychrophilum*, one of the fry trout that is healthy or suspicious of disease found in four different rainbow trout plants located in Keban District of Edirne Province, was practiced through the common culture method. The typology of *F .psychrophilum*, identified by the Polymerase Chain Reaction technique was carried out by the Pulsed Field Gel Electrophoresis technique regarded as the golden standard of molecular typology methods and the affinity between the isolates and *F. psychrophilum* brought from abroad, was determined by the reference strain. In the end, phenotypic and genotypic features of *F. psychrophilum* were found out.

In their studies, Schmidt *et al.* (2000) homogenizing the fish samples in the stomacher, stated that they had prepared those samples for bacteriological culture. In another research (Yıldırım, 2007), the fish material was pestled in porcelain mortar and used for bacterial culture. Because it was more sterile and gave result in a short time, the stomacher homogenizer was also preferred in this research. Accordingly, 1835 eggs in total, eggs disinfected with povidone/iodine solution, fertilized egg, controlled egg, marsupial fry, fry smaller than 5 g and fish food samples taken from four plants where the research was carried out, were homogenized successfully in the stomacher and used for bacteriological culture.

In their studies, Sander and Fryer (1988), Bernardet and Kerouault (1989), Crump *et al.*, (2001) Wiklund *et al.*, (1994), Lorenzen *et al.*, (1997), Bowser (1999), Dalsgaard and Madsen (2000), Korun and Timur (2001), Nematollahi *et al.*, (2003), Diler *et al.*, (2003), Ispir *et al.*, (2004) and Cipriano and Holt (2005); incubed CA, MAOA and TYES-A mediums at 15-20°C for seven days, that they cultured in order to isolate *F. psychrophilum*. In these mediums, mid-fluffy, convex, circular and yellow colonies with thin spreading edges were produced and inside the painted preparates made by these colonies they specified the presence of Gram negative bacilli-shaped, gliding bacteria with 1,20-1,30 µm length. Findings of the researchers relevant to the colony structure and active features of 160 *F. psychrophilum*, isolated from trout plants in Keban district.

It was stated that, the colonies formed by the isolated *F. psychrophilum* in the researches carried out, appeared yellow, the oxidase test was negative, flexirubin-type pigment production test was orange in colour, gelatin and casein hydrolisation test was positive, congo red test and the acid production from H_2S and carbonhydrates was negative (Wiklund *et al.*, 1994; Lorenzen *et al.*, 1997; Madetoja *et al.*, 2001; Cipriano and Holt 2005; Ekman, 2003). In this study, in the identification of *F. psychrophilum*, isolated from the samples of eggs disinfected with povidone/iodine solution, fertilized egg, controlled egg, marsupial fry, fry smaller than 5 g, and from spleen, liver, kidney, guts and water samples of fry trout bigger than 5g, healthy , suspicious of disease or just died, these distinctive biochemical tests were successfully carried out and similar results were acquired.

In similar studies (Diler *et al.*, 2003; Korun and Timur 2001; Didinen *et al.*, 2005), it was reported that the oxidase test was negative, and in some of them (Bernardet and Kerouault 1989; Ostland *et al.*, 1997; Elsayed *et al.*, 2006), it gave positive results. It was determined that all the strains isolated in this study was oxidase negative. This supports the idea of phenotypic differences between *F. psychrophilum* strains.

In the identification of *F. psychrophilum*, Cipriano and Holt (2005) and Didinen *et al.*, (2005) stated that O/F test was negative, İspir *et al.*, (2004) stated that the oxidase test was negative and the fermentation test was positive. In this study, it was found out that the fermentation test was both positive and negative for some strains. It was determined that O/F test of *F .psychrophilum* reference bacteria strain was negative.

In a study (Lorenzen *et al.*, 1997), *F. psychrophilum* did not reproduce at 25 °C, and in another study (Bernardet and Kerouault 1989), again this determinant rarely reproduced at 25 °C and did not reproduce over 25 °C. Lorenzen *et al.*, (1997) stated that 18 *F. psychrophilum* out of 25, rarely reproduced at 25 °C, however did not reproduce at 30 °C, Schmidtke and Carson (1995) stated that 18 isolates out of 20, rarely reproduced at 25 °C and did not reproduce at 30 °C. Finally, Dalsgaard and Madsen (2000) determined that this determinant reproduced at 15, 20 and 25 °C. It was observed that, the research subject, 160 strains isolated from the trout plants in Keban, reproduced at 15, 20 and 25 °C, thus it partially showed similarity with the findings of Dalsgaard and Madsen (2000). And it also showed parallelism with the results of Lorenzen *et al.*, (1997) when it did not reproduce at 25 °C.

Lorenzen *et al.*, (1997), reported that *F. psychrophilum* did not reproduce in 2% NaCl, although it reproduced in 1% NaCl, Diler et al., (2003) reported that this determinant reproduced in 0.5% NaCl, and did not reproduce in 1% and 2% NaCl, and Nematollahi *et al.*, (2003) reported that *F. psychrophilum* reproduced in 0.8-1% and 2% NaCl. Accordingly, it was determined that this bacteria did not reproduce in 2% saltinity or over.

975 samples from plant no .I; 1040 from plant no .II; 1000 from plant no.III; and 1040 from plant no .IV were analyzed. Total 4024 samples taken from these four plants are thought to be sufficient in terms of analyzing the phenotypic and genotypic features of *F. psychrophilum.*

The presence rates of *F. psychrophilum* in trout plants were different. 45 (4.62 %) *F. psychrophilum* were isolated from plant no .I; 30 (2.88%) from plant no .II; 43 (4.3%) from plant no .III; and 42 (4.15%) *F. psychrophilum* were isolated from plant no .IV. However, differences in these rates were not regarded as significant statictically ($P>0.05$, $F_{3,68}=1.05$).

This determinant, causing fishes to get sick through the adverse changes in the environmental conditions, situated in the ovarian fluid of fishes and in the natural flora of the nervous system, (Sarıeyyüpoğlu, 1984; Austin and Austin, 1987; Holt *et al.,* 1993; Gonzalez *et al.*, 1999; Cengizler, 2000; Gonzalez *et al.*, 2001; Nematollahi *et al.,* 2003; Hatha *et al.,* 2005; Arda *et al.,* 2005; Skrodenyte-Arbaciauskiene *et al.,* 2006; Kubilay *et al.,* 2009a; Kubilay *et al.,* 2009b). Determinants, being isolated in both water and fish samples, show parallelism with the results of researchers studying on this subject. *F. psychrophilum* was not isolated from the fish food taken from all four plants. And this claims that *F. psychrophilum* did not pass on fishes through feed.

The isolation of *F. psychrophilum* from the ovarian fluid of rainbow trout spawners on the plants in Keban, showed parallelism with the previous studies (Holt, 1987; Rangdale *et al.,* 1996; Madsen and Dalsgaard, 2008; Kubilay *et al.,* 2009a). Holt (1987), Ekman *et al.,* (1993) and Madetoja *et al.,* (2002) noted that spawners were the carriers of *F. psychrophilum* pathogeny.

Rangdale et al., (1996) could not isolate this bacteria from the sperm. As a result of this study, the isolation of *F. psychrophilum* from the sperms of male pawners could not be carried out as the researcher noted. Isolation of *F. psychrophilum* without any signs of disease on the spawners examined, confirmed the possibility that male spawners on the plants in Keban could be the carrier of this determinant.

The disease that *F. psychrophilum* caused, progressed in different mortality rates when compared to fish species. 50% mortality was observed for Coho Salmon fishes because of this determinant (Holt, 1987). It was reported that the disease occurred on rainbow trout fry after two months they began to feed and the mortality rate was 75% (Lorenzen *et al.,* 1991; Bruno, 1992). In this research, in four plants, the death of fry after 20-50 days they began to feed, confirmed that the disease could occur within the first months of the fry. However, it was observed that the mortality rates in the plants were 20% less in plant no.I; 30% in plant no.II; 35% in plant no.III; and 40% less in plant no.IV than the rates that the researchers noted (Holt, 1987; Lorenzen *et al.,* 1991; Bruno, 1992). It was understood as, there was a relation between the differences in the water quality of plants and the mortality of disease, and the mortality was low in the plants where the water quality was high.

In some studies (Austin, 1992; Bernardet and Kerouault 1989; Bruno, 1992; Bustos *et al.,* 1995; Lorenzen *et al.,* 1991; Toranzo and Barja, 1993; Wiklund *et al.,* 1994; Didinen *et al.,* 2005), they observed that on sick and dead fishes, on ventral cavity, depending on the fluid, bloating, exophthalmos, condensation on the skin, white lesions were shaped on the dorsal and kaudal fin of several fishes and in the further phases of the disease, the kaudal fin was completely ruined, radiuses appeared, the liver and kidney faded and the spleen was grew. Similar results were acquired in the trout plant where the research was carried out.

In previous years, in a study carried out in the plants located in Keban district, on fry rainbow trout *F. psychrophilum* was isolated (İspir *et al.,* 2004). In these studies carried out in different time limes, the isolation of *F. psychrophilum* from the samples of eggs disinfected with povidone/iodine solution, fertilized egg, controlled egg, marsupial fry, fry smaller than 5 g, and from spleen, liver, kidney, guts and water samples of fry trout bigger than 5g, healthy, suspicious of disease or just died, proved that the contamination carried on through this determinant on the plants in this region.

It was stated that the limited number of microorganisms of PCR technique found in pure and mixed cultures, could be identified within a day, thus it was more advantageous than the culture and serologic tests (Lin and Tsen, 1996). The use of specific primer for PCR amplification made identification easier. In this research, the wrong positive reaction of the bacteria was prevented using two different primer pairs; Flavobacterium type specific GYRA-FP1F and GYRA-FP1R; *F. psychrophilum* type specific PSY1 190–206 and PSY2 1278–1262. Conducting this technique along with negative (*E.coli*) and positive controls (*F. psychrophilum* NCIMB 1947^T referans) in each step, proved that PCR technique was successfully carried out.

In their study, Chen et al., (2008), reported the isolation and identification of *F. psychrophilum* from 139 samples taken from two fish species in two different regions, on fifteen different plants. Then, they exposed these isolates to PFGE analysis with SacI enzyme, and in reference to the tape profile formed, they found out a 88% affinity among rainbow trout isolates and 70% among Coho salmon isolates.In his study, Del Corro., (2010); isolated 25

F. psychrophilum from the kidney and spleen of the trout in five different regions on twelve different plants. These isolates were exposed to PFGE analysis with seventeen enzymes (*ApaI, BamHI, BglI, HindIII, KpnI, MluI, NcoI, NheI, NotI, PvuI, SalI, SmaI, SpeI, SphI, StuI, XbaI* ve *XhoI)* and amongst them, *Stul* enzyme, having the best tape profile, was preferred. With regard to the tape profiles formed as a result of PFGE analysis, 80% affinity was found out among the isolates. Arai *et al*., (2007) performed the isolation and identification of 81 *F. psychrophilum* for three fish species in different regions. These isolates were exposed to PFGE analysis with eight enzymes (*BlnI, XhoI HinfI, SmaI, SacII, KpnI* ve *HindIII*)and amongst them, *Blnl* and *Stul* enzymes, having the best tape profile, were preferred. With regard to the tape profiles formed as a result of PFGE analysis, 80% affinity was found out among the isolates. In this study, it was found out that there was 80% affinity between *F. psychrophilum* strains and reference strains isolated in different plants. The plants situated in the same region and water resources having the same origin, could explain the similar profiles formed by *F. psychrophilum* strains isolated in the plants. In addition, enzyme preferences might also have a role acquiring these results.

In conclusion, in this study, the isolation and identification of *F. psychrophilum,* causing disease on rainbow trout in our country and many countries in the world, especially causing enormous losses of fry trout and the determinant of Psychrophylos, was practiced through the common culture method. However, the isolation of this determinant is difficult and it takes a long time. Moreover, the isolated *F. psychrophilum* can be kept in a refrigerator or deep freezer for a short while. Therefore, using PCR, a molecular technique delivering quick results on the diagnosis of bacterial diseases, the identification of *F. psychrophilum* as molecular was performed for the first time in our country. Thus, the diagnosis of *F. psychrophilum* produced by the culture, through PCR technique in a short time, and the timely treatment, huge financial losses would be prevented in fishing plants.

In this study, the sub-typology of *F. psychrophilum* through Pulsed Field Gel Electrophoresis technique was successfully performed. The success of this study will cast light upon more original and comprehensive future studies in aquaculture sector towards the epidemiology of fish disease determinants observed in all our regions.

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References

1. Arai, H., Morita, Y., Izumi, S., Katagiri, T., Kimura, H., 2007. Molecular typing by pulsed-field gel electrophoresis of *Flavobacterium psychrophilum* isolates derived from Japanese fish. J Fish Dis.:30(6):345–55.
2. Arda, M., 2000. Temel Mikrobiyoloji; "Genişletilmiş İkinci Baskı.. Medisan Yayın Serisi no 46. Ankara".
3. Arda, M., Seçer, S., Sarıeyyüpoğlu, M., 2005. Balık Hastalıkları Medisan Yayın serisi: 61, II. Baskı Ankara 230s.
4. Austin, B and Austin, D.A., 1987. Bacterial Fish Pathogens: Disease in Farmed and Wild Fish. Ellis Horwood Ltd. Chichester, 363s.
5. Austin, B., 1992. The recovery of *Cytophaga psychrophila* from Two Cases of Rainbow Trout (*Oncorhynchus mykiss*, Walbaum) Fry Syndrome in the U.K. Bull. Eur. Assoc. Fish Pathol. 12: 207–208.
6. Austin, B., and Austin, D. A. 1999. Fish Pathogens Disease in Farmed and Wild Fish, 3rd ed. (revised), Praxis Publ., Chiester, UK.
7. Balta, F., 1997. Kültürü yapılan alabalıklarda *(Oncorhynhus mykiss)* görülen *Flexibacter psychrophila* enfeksiyonu. IX. Ulusal Su Ürünleri Sempozyumu Eğirdir/İsparta, 641–648.
8. Bej, A.K., Mahbubani M.H., Atlas, R.M., 1991. Amplification of Nucleic Acids by Polymerase Chain Reaction (PZR) and Other Method and Their Amplications. Critical Reviews in Biochemistry and molecular Biology, 26, 301–334.
9. Bernardet, J.F. and Keroualt, B., 1989. Phenotypic and Genomic Studies of ‘‘*Cytophaga psychrophila*’’, Isolated from Diseased Rainbow Trout (*Oncorhynhus mykiss*) in France. Appl. Environ. Microbial. Vol. 55, No: 7, 1796–1800.
10. Bernardet, J.F., Segers, P., Vancanneyt, M., Berthe, F., Kersters, K. and Vandamme, P., 1996. Cutting a Gordian Knot: Emended Classification and Description of the Genus Flavobacterium, Emended Description of the Family Flavobacteriaceae, and Proposal of *Flavobacterium hydatis* nom. nov. (Basonym, *Cytophaga aquatilis* Strohl and Tait 1978) Internatıonal Journal Of Systematıc Bacterıology, Jan. 128–148.
11. Bowser, P.R., 1999. “Diseases of Fish”, PhD, Aquatic Animal Health Program, Department of Microbiology and Immunology, College of Veterinary Medicine Cornell University, Ithaca, New York, 26 p.
12. Bruno, D.W., 1992. “*Cytophaga psychrophila*, (*Flexibacter psychrophilus*)(Borg), histophathology associated with mortalities among farmed rainbow trout *(Oncorhynchus mykiss*) (Walbaum) in the UK ”, Bull. Eur. Ass. Fish Pathol., 12(6):215-216.
13. Buckley, R., Clough, E., Warken, W., Wıld, C., 1998. Coliform Bacteria in Streambed Sediments in a Subtropical Rainforest Conservation Reserve. Wat.Res., 32(6), 1852-1856.
14. Bustos, P., Calbuyahue, A., Maontaña, J., Opazo, B., Entrala, P. and Solervicens, R., 1995. First isolation of *Flavobacterium psychrophilum* as causative agent of rainbow trout fry syndrome (RTFS) in Chile. Bull. Eur. Assoc. Fish Pathol. 15: 162–164.
15. CDC, 2010. One-Day (24-28 h) Standardized Laboratory Protocol for Molecular Subtyping of *Escherichia coli* O157:H7, non-typhoidal *Salmonella* Serotypes, and *Shigella sonnei* by Pulsed Field Gel Electrophoresis (PFGE). Erişim: (http://www.cdc.gov/PULSENET/protocols/ecoli_salmonella_shigella_protocols.pdf). Erişim tarihi: 28.06.2010.
16. Cengizler, İ., 2000. Balık Hastalıkları Ders Kitabı. Çukurova Ünivesitesi Su Ürünleri Fakültesi Yayınları No: 7, 136s.
17. Chen, Y.C., Davis, M.A., Lapatra, S.E., Cain, K.D., Snekvik, K.R., Call, D.R., 2008. Genetic diversity of *Flavobacterium psychrophilum* recovered from commercially raised rainbow trout, *Oncorhynchus mykiss* (Walbaum), and spawning coho salmon, O. kisutch (Walbaum) J Fish Dis. :31(10):765-73.

18. Cipriano, R.C. and Holt R.A., 2005. *Flavobacterium psychrophilum*, Cause of Bacterial Cold- Water Disease and Rainbow Trout Fry Syndrome. Fish Disease Leaflet No:86. United States Dept. of the Interior. U.S. Geological Service, National Fish Health Research Laboratory, Kearneysville, WV. 44s.
19. Crump, E.M., Perry, M.B., Clouthıer, S.C., Kay, W.W., 2001. Antigenic Characterization of the Fish Pathogen *Flavobacterium psychrophilum*, Applıed and environmental microbiology, 67, 750-759.
20. Dalsgaard, I. and Madsen, L., 2000. Bacterial pathogens in rainbow trout *Oncorhynchus mykiss* (Walbaum), reared at Danish freshwater farms. J. Fish Dis. 23: 199–209.
21. Daskalov, H., Austin, D.A., Austin B., 1999. An improved growth medium for *Flavobacterium psychrophilum*. Letters in Applied Microbiology 28: 297–299.
22. Del Cerro, A., Márquez I., Prieto J.M., 2010. Genetic diversity and antimicrobial resistance of *Flavobacterium psychrophilum* isolated from cultured rainbow trout, *Onchorynchus mykiss* (Walbaum), in Spain. J Fish Dis. :33(4):285–91.
23. Derbentli Ş., 2002. Hastane infeksiyonlarının epidemiyolojisinde moleküler yöntemlerin yeri. Ağaçfidan A, Badur S, Türkoğlu S (Editörler). İnfeksiyon hastalıklarının laboratuar tanısında moleküler yöntemler'inde. İstanbul: Türk mikrobiyoloji cemiyeti yayın; 6–13.
24. Didinen, B.I., Diler, Ö., Ekici, S., Altun, S., 2005. *Flavobacterium psychrophilum* izolatlarının teşhisinde API ZYM kullanımı ve ATB VET ile antimikrobiyal duyarlılığın belirlenmesi, Süleyman Demirel Üniversitesi Eğirdir Su Ürünleri Fakültesi Dergisi, 1 (2): 64-70.
25. Diler, Ö., Altun, S., Çalıkuşu, F., Diler, A., 2000. Gökkuşağı alabalığı (*Oncorhynchus mykiss*)'nin yaşadığı ortam ile ilişkili kalitatif ve kantitatif bakteriyel florası üzerine bir araştırma. Turk J. Vet. Anim. Sci. 24: 251–259
26. Diler, Ö., Altun, S., Işıklı, B.I., 2003. Kültürü yapılan gökkuşağı alabalığı (*Onchorhynchus mykiss*)'ndan izole edilen *Flavobacteriun psychrophilum*'un fenotipik karakterleri. Süleyman Demirel Üniversitesi Fen Bilimleri Enstitüsü Dergisi, 7.1, 1–8.
27. Domig, J.K., Mayer, HK., Kneifel, W., 2003. Methods used for the isolation, enumeration, characterisation and identification of *Enterococcus* spp.2. Pheno- and genotypic criteria. International Journal of Food Microbiology.; 88 :165–188.
28. Ekman, E., 2003. Natural and experimental Infections with *Flavobacterium psychrophilum* in salmonid fish. Swedish University of agricultural sciences, Doctoral thesis, 47s.
29. Ekman, E., Börjeson, H., Johansson, N., 1993. *Flavobacterium psychrophilum* in Baltic *salmon Salmo salar* brood fish and their offspring. Dis Aquat Org, 37, 159-163.
30. Elsayed, E.E., Eissa, A.E., Faisal, M., 2006. Isolation of *Flavobacterium psychrophilum* from sea lamprey, *Petromyzon marinus* L., with skin lesion in Lake Ontairo, Journal of Fish Diseases, 29: 629-632.
31. Gonzalez, C.J., Lopez-Diaz, T.M., Garcia-Lopez, M.L., Prieto, M., Otero, M., 1999. Bacterial microflora of wild brown trout (*Salmo trutta*), wild pike (*Esox lucius*) and aquacultured rainbow trout (*Oncorhynchus mykiss*), Journal of Food Protection, 62(11): 1270-1277.
32. Gonzalez, C.J., Santos, J.A., Garcia-Lopez, M.L., Gonzalez, N., Otero, A., 2001. *Mesophilic aeromonads* in wild and aquacultured freshwater fish, Journal of Food Protection, 64(5): 687-691.
33. Hatha, M., Vivekanandhan, A.A., Joice, G.J., Christol, J.J., 2005. Antibiotic resistance pattern of motile aeromonads from farm raised fresh water fish, International Journal of Food Microbiology, 98(2):131-134.
34. Holt, R.A., 1987. Cytophag, Psychrophil, the Causative Agent of Bacterial Cold-Water Disease in Salmonid Fish Oregon State University A Thesis 182 p.
35. Holt, R.A., Rohovec, J.S. and Fryer, J.L., 1993. " Bacterial cold-water disease ", In: "Bacterial Diseases of Fish ", Inglis, V., Roberts, R. and Bromage, N. R. (ed.), Blackwell Scientific Publications, Oxford., 312p.
36. İspir, Ü., Şeker, E., Sağlam, N., Dörücü, M., 2004. Doğu Anadolu bölgesinde bazı gökkuşağı alabalığı (*Oncorhynchus mykiss*) işletmelerinde görülen *Flavobacterium psychrophilum* enfeksiyonunun araştırılması, Fırat Üniversitesi Fen ve Mühendislik Bilimler dergisi, 16 (4), 718-724.
37. Korun, J. ve Timur, G., 2001. Gökkuşağı Alabalıklarında (*Onchorhynchus mykiss*) fry mortalite sendromu (FMS) üzerinde bir çalışma İstanbul Üniversitesi su ürünleri dergisi 12:15-30.
38. Kubilay A., Altun, S., Savaş S., 2009b. A study on aerobic bacterial flora during incubation of rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792) eggs in hatchery. Journal of FisheriesSciences. 3(1) 5–9.
39. Kubilay, A., Altun, S., Didinen, B.I., Ekici, S., Diler, Ö., 2009a. Gökkuşağı Alabalığı (*Oncorhyncus mykiss*) İşletmelerinde *Flavobacterium psychrophilum* İzalosyonu Kafkas Univ. Vet. Fak. Derg. 15 (5): 709–715.
40. Kumagai, A., Nakayasu, C., Oseko, N., 2004. Effect of Tobramycin Supplementation to Medium on Isolation of *Flavobacterium psychrophilum* from Ayu *Plecoglossus altivelis* Japan Science and Technology Agency 39;2; 75-78
41. Lin, C.K., and Tsen H.Y., 1996. Use of two 16S DNA Targeted Oligonücleotides as PZR Primers for the Specific Detection of Salmonella in Foods. Journal of Applied Bacteriology, 80: 659–666.
42. Lorenzen, E., 1994. Studies on *Flexibacter psychrophilus* in relation to rainbow trout fry syndrome (RTFS), National Veterinary Laboratory, Royal Veterinary and Agricultural University, Copenhagen.
43. Lorenzen, E., Dalsgaard, I., Bernardet, J.F., 1997. Characterization of isolates of *Flavobacterium psychrophilum* associated with coldwater disease or rainbow trout fry syndrome I: phenotypic and genomic studies, Diseases of Aquatic Organisms, 31: 197-208.
44. Lorenzen, E., Dalsgaard, I., From, J., Hansen, F. M., Horlyck, V., Korsholm, H., Mellergaard, S. and Olesen, N.J., 1991. Preliminary investigations of fry mortality syndrome in rainbow trout. Bull. Eur. Assoc. Fish Pathol. 11: 77–79.
45. Lumsden, J.S., Ostland, V.E. and Ferguson, H.W., 1996. Necrotic Myositis in Cage Cultured Rainbow Trout, *Oncorhynhus mykiss* (Walbaum), Caused by *Flexibacter psychrophilus*. Journal of fish diseases 19; 113–119.
46. Madetoja, J. and Wiklund, T., 2002. Detection of the fish pathogen *Flavobacterium psychrophilum* in water from fish farms. System. Appl. Microbiol. 25: 259–266.
47. Madetoja, J., Dalsgaard, I., Wiklund, T., 2002. Occurrence of *Flavobacterium psychrophilum* in fish-farming environments. Dis Aqua Org, 52, 109-118.
48. Madetoja, J., Hänninen, M-N., Hirvelä-Koski, V., Dalsgaard, I. and Wiklund, T., 2001. Phenotypic and genotypic characterization of *Flavobacterium psychrophilum* from Finnish fish farms. J. Fish Dis. 24:469–479.
49. Madsen, L and Dalsgaard, I., 2008. Water recirculation and good management potential methods to avoid disease outbreaks with *Flavobacterium psychrophilum*. J Fish Dis, 31, 799-810,
50. Maslow, J.N., Slutsky, A.M. and Arbeit, R.D., 1993. Application of PFGE to molecular epidemiology. P: 563–572. Diagnostic Molecular Microbiology: Principles and Applications. ASM Washington D.C.
51. Mullis, K.B., 1990. The Unusual Origin of the Polymerase Chain Reaction. Sci. Am. 262 (4) : 56 – 65.
52. Nematollahi, A ., Decostere, A ., Pasmans, F. and Haesebrouck, F., 2003. *Flavobacterium psychrophilum* infections in salmonid fish. Journal of Fish Diseases, 26: 563–574.
53. Ostland, V.E., McGrogan, D.G., Ferguson, H.W., 1997. Cephalic osteochondritis and necrotic scleritis in intensively

reared salmonids associated with Flexibacter psychrophilus, Journal of Fish Diseases, 20: 443-451.
54. Pacha, 1968. Characteristics of *Cytophaga psychrophila* (Borg) Isolated During Outbreaks of Bacterial Cold-Water Disease, Applıed Mıcrobıology, p. 97-101
55. Rangdale, R.E., Richards R.E., Alderman, D.J., 1996. Isolation of *Cytophaga psychrophila*, causal agent of rainbow trout fry syndrome (RTFS) from reproductive fluids and egg surfaces of rainbow trout (*Oncorhynchus mykiss*). Bull Eur Ass Fish Pathol 16, 63-67.
56. Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. and Erlich, H.A., 1988. Primer-Directed Enzymatic Amplification of DNA with a Thermostable DNA Polymerase. Science; 239, 487–491.
57. Sander, J.E., Fryer J.L., 1988. Bacteria of Fish in "Methods in Aquatic Bacteriology" (Ed by AUSTIN), John wiley & Sons ltd., Cichester, New York, Brisbane, Toronto, Singapore.
58. Sarıeyyüpoğlu, M., 1984. Gökuşağı Alabalıklarında (*S. gairdneri*) Mide-Bağırsak Bakteriyel Florasının Aerobik Yönden İncelenmesi. Doğa Bilim Dergisi, Seri: D 1 Cilt: 8, Sayı: 3, S. 281-287.
59. Schmidt, A.S., Bruun, M.S., Dalsgaard, I., Pedersen, K. and Larsen, J.L., 2000. Occurrence of antimicrobial resistance in fish-pathogenic and environmental bacteria associated with four Danish rainbow trout farms. Applied and Environmental Microbiology 66, 4908–4915.
60. Schmidtke, L.M., Carson, J., 1995. Characteristics of *Flexibacter psychrophilus* isolated from Atlantic salmon in Australia, Diseases of Aquatic Organisms, 21:157-161.
61. Skrodenyte-Arbaciauskiene, V., Sruoga, A., Butkauskas, D., 2006. Assessment of microbial diversity in the river trout, *Salmo trutta fario L.*, intestinal tract identified by partial 16SrRNA gene sequence Analysis. Fisheries Science, 72(3): 597–602.
62. Starliper, C., Marcquenski S., Noyes A., 2007. Development of an improved medium for primary isolation of *Flavobacterium psychrophilum*, cause of bacterial coldwater disease.
63. Suzuki, K., Arai, H., Kuge, T., Katagiri, T., İzumi S., 2008. Reliability of PCR Methods fort he Detection *Flavobacterium psychrophilum*. Fish Patology, 43 (3), 124-127
64. Taylor, G.R., 1993. Polymerase Chain Reaction: Basic Principles and Automation.In PZR: A Pratical Approach, Eds:McPherson, M.J., Quirke, P. and Taylor, G.R.Oxford University Press,Oxford, 1-14.
65. Toranzo, A.E. and Barja, L.J., 1993. "Fry Mortality Syndrome (FMS) In Spain. Isolation of The Causative Bacterium *Flexibacter psychrophilus*", Bull. Eur. Ass. Fish Pathol., 13(1):30-32.
66. Toshio, N., Yuichi, I., Hajime, A., Takahiro N., Shotaro, I., 2006. A Newly Designed Culture Medium for *Flavobacterium psychrophilum* Japan Science and Technology Agency 28;.33-37
67. Van Belkum, A., Tassios, P.T., Dijkshoorn, L., Haeggman, S., Cookson, B., Fry, NK., Fussing, V., Gren, J., Feil, E., Gerner-Smidt, P., Brisse, S., Struelens, M., 2007. Guidelines for the validation and applicationof typing methods for use in bacterial epidemiology. Clinical Microbiology and Infectious Diseases.; 13: 1–46
68. Vastos, I.N., Thompson, K.D. and Adams, A., 2003. Starvation of *Flavobacterium psychrophilum* in broth, stream water and distilled water. Dis. Aquat. Org. 56: 115–126.
69. Wiklund, T., Kaas, K., Lönnström, L., Dalsgaard, I., 1994. Isolation of *Cytophaga psychorphila* (*Flexibacter psychorphilus*) from wild and farmed rainbow trout (*Oncorhynchus mykiss*) in Finland, Bulletin of the European Association of Fish Pathologists, 14 (2): 44-46.
70. Yıldırım, S., 2007. Mersin Çağlarca Köyündeki Gökkuşağı Alabalığı (*Oncorhynchus mykiss*,Walbaum, 1792) Kuluçkahanelerinde *Flavobacterum* spp.'nin Araştırılması. Mersin Üniversitesi Fen Bilimleri Enstitüsü Yüksek Lisans Tezi 79s.
71. Barrett T.J., Lior H., Green J.H., Khakhria R., Wells J.G., Bell B.P., Greene K.D., Lewis J. & Griffin P.M. (1994) Laboratory investigation of a multistate food-borne outbreak of Escherichia coli O157:H7 by using pulsed-field gel electrophoresis and phage typing. Journal of Clinical Microbiology 32,
72. 3013–3017.
73. Izumiya H., Terajima J., Wada A., Inagaki Y., Itoh K., Tamura K. & Watanabe H. (1997) Molecular typing of enterohemorrhagic Escherichia coli O157:H7 isolates in Japan by using pulsed-field gel electrophoresis. Journal of Clinical Microbiology 35, 1675–1680.