Alterations in Erythrocyte Osmotic Fragility and Erythrocyte Membrane Fatty Acid Profile of Rainbow Trout (*Oncorhynchus mykiss*) Experimentally Infected with *Aeromonas salmonicida*

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Abstract: Erythrocyte osmotic fragility and erythrocyte membrane fatty acid profiles of Rainbow trout (*Oncorhynchus mykiss*) experimentally infected with *Aeromonas salmonicida* were investigated at different days of injection. Erythrocytes of the infected fish were found more fragile than the controls. While 50% of haemolysis of diseased fish erythrocytes occurred at chloride concentration of 0.49±0.01%, it was 0.40±0.01% for controls. Three remarkable trends in erythrocyte fatty acid patterns in infected fish observed when compared with the controls. While no significant differences in saturated fatty acids were detected significant decrease in monounsaturated acids and increase in polyunsaturated fatty acids were also detected.

Key words: Erythrocyte, osmotic fragility, membrane fatty acids, *Aeromonas salmonicida*, rainbow trout (*Oncorhynchus mykiss*)

INTRODUCTION

Although, aquatic environment and fish are relatively rich in microorganisms in most cases fish can survive without any problems but many bacterial genera have at various times been described as pathogens of both freshwater and marine fish species. Mortality in fish stocks due to a bacterial infection only take place when pathogenic bacteria and fish are available in an environment that results with disease (Austin and Austin, 1987; Gudmundsdottir and Bjornsdottir, 2007).

Because of its popularity in fish disease, *Aeromonas salmonicida* has been studied more than any other fish pathogen. *Aeromonas salmonicida* ssp. *salmonicida* is the causative agent of classical frunkulosis a highly fatal epizootic disease of salmonid fishes is called typical but other subspecies like ssp. *achromogenes, masoucida smithia* and *pectinolytica* are called atypical and new isolates that don't fit into the existing classification are reported worldwide and the taxonomy of the genus Aeromonas appears to be continuously changing due to the addition of newly described species. Simply, an atypical strain can be defined as a strain that does not fit into the existing classification of *Aeromonas salmonicida*

ssp. *salmonicida*. This atypical strains are known to cause ulcerative or generalized diseases in a wide variety of both freshwater and marine fish (Austin and Austin, 1987; Gudmundsdottir, 1998; Pavan *et al.*, 2000; Beaz-Hidalgo *et al.*, 2009).

Red Blood Cells (RBCs) also referred to as erythrocytes are the most common type of blood cell. Oxygen and carbon dioxide transport within the blood is related to the electrolytes and also acid-base status of the red blood cells, this transport depends on the permeability of the erythrocyte membrane. Membrane fluidity is strongly effected by fatty acids especially those in ester phospholipids control the structure and function of biological membranes (Thomas and Egee, 1998; An *et al.*, 2009).

The degree of resistance of red blood cells to lysis as a result of a decrease in the NaCl content of their environment is the basis of the osmotic fragility test. The degree of hemolysis is determined by measurement of hemoglobin release from the cells. The osmotic fragility test is used to determine the extent of red blood haemolysis produced by osmotic stress. Although, an old method, the osmotic fragility test is a useful screening assay to detect several pathological conditions and

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clinically as a diagnostic property (Orcutt *et al.*, 1995; Kafka and Yermiahu, 1998). The aim of this study was to determine the effects of *Aeromonas salmonicida* infection on osmotic fragility and also membrane fatty acids of rainbow trout (*Oncorhynchus mykiss*) erythrocytes.

MATERIALS AND METHODS

Fish and facilities: This study was conducted in Fisheries Department of Agricultural Faculty at Ataturk University (Erzurum/Turkey). A total of 50 rainbow trout (*Oncorhynchus mykiss*) weighting 250±45 g were obtained from a freshwater farm. Fish were randomly divided into two groups (25 fish in each tank) one for control and the other for experimental infection. Fish were kept in two separate 300, l circular fiberglass tanks with a constant water flow of 1.5l min⁻¹ of aerated dechlorinated tap water and held under natural light conditions. During the entire experimental period the water in the tanks had the following characteristics: temperature 9-11°C; dissolved oxygen 8-9 ppm; pH 7.6-7.8. Fish were acclimated to these conditions for 2 weeks before the start of the experiment. Fish were fed pelleted dry food (40% protein) at a rate of 1.5% body weight day⁻¹. Tanks were cleaned daily by siphoning.

Bacteria and identification: Atypical *Aeromonas salmonicida* (A-480) strain used in the experiment was obtained from the Central Fisheries Research Institute (Trabzon, Turkey). Firstly, lyophilized bacteria were grown on Tryptic Soy Broth (TSB) than Transferred to tryptic Soy Agar (TSA) plates supplemented with NaCl to a final concentration of 1% at 25°C for 24 h (Beaz-Hidalgo *et al.*, 2010). After incubation, isolates were identified by Fatty Acid Methyl-Ester (FAME) gas chromatography analysis using Microbial Identification Systems Software (MIS Delaware, USA). Strains were fractionally streaked in four quadrants of a plate containing TSA. The growth from the third quadrant was harvested and placed in a glass tube (5 mL) with a screw cap. After saponification, methylation, extraction and base wash processes the tubes left room temperature for 5 min. About 2 mL of the top phases were transferred to chromatography tubes using a multichannel pipette. Specimens were processed on a MIDI Sherlock® Microbial Identification System with a Hewlett-Packard automatic sampler and integrator. For identification, clinical aerobic library was used (Buyer, 2002).

Experimental infection: After an 24-72 h incubation period at 25°C in TSA, *A. salmonicida* colonies were aseptically removed and suspended in Phosphate-Buffered Saline (PBS) solution (0.001M PBS, Ph 7.4) in order to obtain approximately 10⁶CFU (colony forming units). About 0.5 mL of this solution was injected intramuscularly anterior to the dorsal fin. Non infected control group was injected with the same amount of PBS.

Blood sampling, determination of osmotic fragility and fatty acid composition: In order to compare daily alterations of the osmotic fragility and erythrocyte membrane fatty acid composition, blood samples were taken 1, 3, 7, 14 and 21 days after the start of the experiment. A total of 10 fish were sampled (5 control, 5 infected) at each day. To avoid sampling stress, fish were anaesthetized with MS 222 (Sigma Chemical Co.). Blood samples were drawn from each fish by caudal venous puncture with a heparinized disposable sterile syringe. Osmotic fragility of the fish erythrocyte was determined according to procedure of Aldrich and Saunders. In brief, 20 µL of each blood sample was subjected to a series of 1 mL NaCl solutions ranging from 0.85% NaCl concentration to 0.0% NaCl (distilled water) were used. After an incubation period of 30 min at room temperature the solutions were centrifuged for 5 min at 2000 rpm. The optical density of the supernatant was then determined spectrophotometrically at 540 nm. The percentage hemolysis was expressed relative to the solution having the highest optical density reading then graphs were made for each blood sample by comparing hemolysis and NaCl concentration and from this curves the percent NaCl concentration at 50% hemolysis for each individual was determined. For fatty acid determination of the erythrocyte membrane of the fish, 1.5 mL of blood samples were centrifuged and erythrocytes was hemolyzed with distilled water 3 times and this membranes were analyzed by a gas chromatograph (Hisar *et al.*, 2003).

Statistical analysis: The means and standard deviations of means for each parameter were calculated. Variance analysis was performed to evaluate changes in daily variables between control and infected fish. In cases where a p<0.05 was found Duncan's multiple range test was performed for over time changes in blood values. All the calculations were carried out using the statistical package SPSS for Windows.

RESULTS AND DISCUSSION

Although, a low mortality rate (8%) occurred in *A. salmonicida* infected group (two fish died 21 days after injection) pure culture of *A. salmonicida* could be isolated from the all diseased fish especially from the kidney. Bacteria also isolated from the skin lesions but this were

not pure cultures, Furones (2001) reported that the real pathogen can be overgrown by opportunistic bacteria when the pathogen produces an external disease condition. Pathological and clinical findings observed in some of the fish from the infected group were skin lesions that filled with turbid exudates, pale gills, hemorrhages especially at the site of injection, extensive erosion of the pectoral and caudal fins, turbid fluid within the abdominal cavity, brown pigmentation on the skin, almost all of the fish in the infected group hang just under the surface of the water and breathe rapidly some of the fishes eyes in the infected group become cloudy. No mortalities and clinical signs of a bacterial disease were recorded in the control group. Because of pathological process did not reach deeper layers, fruncules seen in frunkulosis does not observed any of the infected fish (Rehulka, 2002). Fatty acids profile of the bacteria were used for the bacterial identification in the present study. Total 7 different fatty acids were identified in the bacterial lipid extracts. Among this, major fatty acids for *A. salmonicida* determined as 16:1 w7c/15 iso 2OH (46.12%), 18:1 w7c

(8.33%) monounsaturated fatty acids and 14:0 3OH/16:1 iso I (8.83%), 16:0 (23.75%) saturated fatty acids. Krejci *et al.* (2009) reported major fatty acids for *A. salmonicida* as palmitoleic acid (39.4%), palmitic acid (18.1%) and oleic acid (17.8%). Bogdan *et al.* (2001) also reported predominance of palmitic acid, palmitoleic acid and oleic acid for motile and nonmotile aeromonads.

For an identification of a bacteria, the presence of distinct fatty acids and their relative amount is analyzed and compared with the fatty acid profiles of reference strains and also this is one of the most developed chemotaxonomic method for the identification of a bacteria. Biochemical, nutritional and physiological characterization tests are time consuming and laborious tests (Busse *et al.*, 1996). Successful treatment of a bacterial disease requires an early and accurate diagnosis, if diagnosis and also treatment of a bacterial disease is delayed, this situation can cause substantial mortality of the fish stocks. Osmotic fragility curves are shown in Fig. 1. In osmotic fragility studies at the 1st, 3rd, 7th,
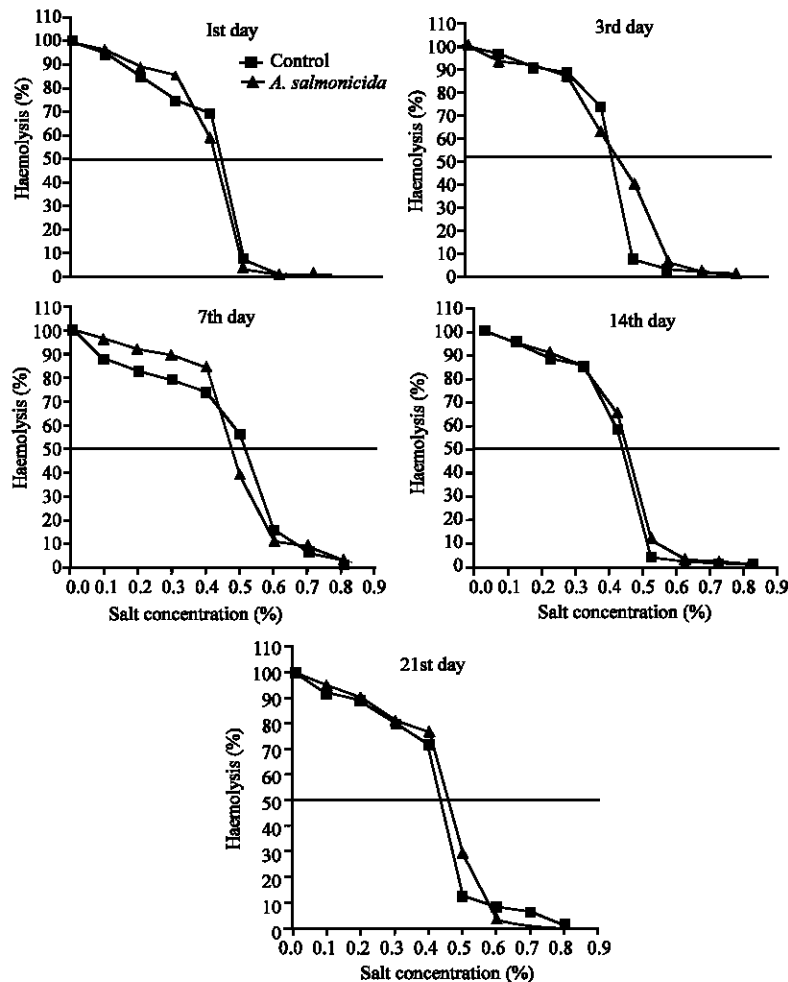

Fig. 1: Daily alterations of erythrocyte osmotic fragility in healthy and *P. putida*-infected

Table 1: Daily alterations in erythrocyte membrane fatty acid composition in control and *A. salmonicida* infected fish

Fatty acids	Groups	Days				
		1	3	7	14	21
16:0	Control	31.0±0.170	28.9±1.200	27.37±0.80	27.8±0.200	28.6±0.480
(PA)	Diseased	27.98±2.58	28.74±0.89	28.45±4.00	30.30±0.06	28.55±0.77
18:0	Control	7.23±2.94	10.6±0.600	7.29±1.85	5.6±0.440	7.0±1.470
(SA)	Diseased	7.87±2.04	7.58±0.45	6.83±1.24	8.41±0.24	10.96±0.91
18:1w9c	Control	10.65±0.91	8.9±0.900	11.20±0.84	13.9±0.300	9.1±1.860
(OA)	Diseased	9.02±0.14	6.46±0.11	8.95±0.80	8.22±0.46	6.21±0.56
18:2w6c	Control	7.14±2.94	6.4±0.040	7.02±0.29	6.8±0.310	6.7±0.430
(LA)	Diseased	6.02±0.96	5.37±0.23	5.82±0.91	6.92±0.96	5.12±0.19
20:4w6c	Control	3.12±0.53	5.4±0.010	3.98±0.04	3.8±0.070	5.1±0.190
(AA)	Diseased	6.10±1.21	5.71±0.12	5.42±0.24	6.01±0.13	5.43±0.12
20:5w3c	Control	5.65±1.52	5.5±0.300	6.15±1.20	5.1±0.130	7.2±0.330
(EA)	Diseased	6.13±1.32	5.44±0.65	6.31±0.18	5.75±0.16	6.12±0.12
22:6w3c	Control	34.86±4.05	33.6±0.500	32.20±0.56	32.9±0.500	35.2±1.390
(DA)	Diseased	36.87±1.25	38.67±1.51	37.73±5.86	34.14±0.68	35.89±0.77
Total	Control	99.65	99.7	95.21	95.9	98.9
	Diseased	99.99	97.97	99.51	99.75	98.28
SFA	Control	38.24±2.77	39.52±0.58	34.66±1.04	33.40±0.17	35.63±1.95
	Diseased	35.85±4.63	36.33±0.43	35.28±5.24	38.72±0.21	39.51±0.86
MUFA	Control	10.65±0.91	8.92±0.92	11.20±0.84	13.97±0.32	9.19±1.86
	Diseased	9.02±0.14[a]	6.46±0.11[b]	8.95±0.80[c]	8.22±0.46[c]	6.21±0.56[b]
PUFA	Control	49.28±0.03	51.10±0.86	49.36±1.52	48.67±1.08	54.31±1.29
	Diseased	55.12±4.76[a]	55.20±2.27[a]	55.28±5.38[a]	52.45±0.97[b]	52.57±0.97[b]

Results are expressed as mean±SD. PA, Palmitic Acid; SA, Stearic Acid; OA, Oleic Acid; LA, Linoleic Acid; AA, Arachidonic Acid; EA, Eicosapentaenoic Acid; DA, Docosahexaenoic Acid; SFA, Total saturated; MUFA, total Monounsaturated; PUFA, total Polyunsaturated fatty acids. Different superscript letters denote statistically significant differences between total fatty acids of control and diseased fish at $p<0.05$

14th and 21st days of the experimental infection trial, 50% of haemolysis of fish erythrocytes occurred at NaCl concentrations of 0.49±0.03, 0.51±0.04, 0.44±0.06, 0.43±0.01 and 0.40±0.01%, respectively in control fish and 0.43±009, 0.48±0.06, 0.42±0.02, 0.46±0.04 and 0.49±0.01% in the *Aeromonas salmonicida* injected fish. These results indicate that significant increases in erythrocyte osmotic fragility between the controls and infected fish only occurred on the first and last days of the study, erythrocytes of the infected fish were more fragile and the chloride concentration was 0.49±0.01% on the last day of the experiment.

Barham *et al.* (1980) reported that *Streptococcus* and *Aeromonas* infections in rainbow trout resulted in more fragile erythrocytes and they assumed that these findings could be caused from production of hemolytic factor by bacteria with a resultant disturbance in the functional state of the blood cell membrane. When studying the effects of *Pseudomonas putida* infection on osmotic fragility in rainbow trout (*Oncorhynchus mykiss*), Bektas and Ayik (2009) reported more fragile erythrocyte in infected fish.

Table 1 shown the daily alterations in fatty acids composition of the erythrocyte membrane of the control and *A. salmonicida* infected fish. The fatty acid pattern in infected fish showed three remarkable trends when compared with the control group while no significant differences in saturated fatty acids (Palmitic and Stearic acids) were detected, statistically significant decrease in monounsaturated fatty acid (Oleic acid) and increase in polyunsaturated fatty acids (Linoleic, arachidonic and eicosapentaenoic acids) were also detected.

Phospholipids with proteins are very important functional components of biological membranes. Properties of the phospholipids is mainly determined by their fatty acid composition and it is known that fish biological membranes are rich in polyunsaturated fatty acids and would be sensitive to oxidative stress during their lives fish are exposed to different kinds of stressors like as disease (Orly and Schramm, 1975; Tanaka *et al.*, 2002; Nagasaka *et al.*, 2004; Marcogliese *et al.*, 2005).

Tanaka *et al.* (2002) reported a decrease in polyunsaturated fatty acids in the tissue of different diseased fish species and they claimed that the decreased levels of polyunsaturated fatty acids found in the diseased fish versus the normal fish support enhanced oxidative conditions in diseased fish.

CONCLUSION

Early and accurate diagnosis is important in bacterial fish disease for a successful treatment and this requires the identification of the bacteria as early as possible. The analysis of fatty acid patterns of the bacteria for identification in the present study found much more

effective than the other identification methods. Experimental *Aeromonas salmonicida* infection in rainbow trout induced significant changes both in erythrocyte osmotic fragility and membrane fatty acid profiles, infected fish erythrocytes found more fragile than the controls, significant decrease in monounsaturated fatty acid and increase in polyunsaturated fatty acids were also detected in diseased fish. Interpreted data can provide useful information on *A. salmonicida* disease process or could be used to assess an abnormality in fish life.

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