ORİJİNAL ARAŞTIRMA ORIGINAL RESEARCH

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Investigation of *Aeromonas salmonicida* from Fish Species Living in Lake Sazlıdere, İstanbul

Sazlıdere Baraj Gölünde Yaşayan Balık Türlerinde *Aeromonas salmonicida* Varlığının Araştırılması

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This study was presented in the 18th International Veterinary Medicine Students Scientific Research Congress, Istanbul, Turkey, 26-28 April, 2016 and presented in Ecology2017 Symposium, Kayseri, Turkey, 10-13 May, 2017.

ABSTRACT Objective: The aim of this study was, to examine the presence of *Aeromonas salmonicida* in lake water and fish species living in Lake Sazlıdere, a region where fish hunting was also conducted, in Istanbul. **Material and Methods:** In this study, between April 2014 and March 2015, samples from Lake Sazlıdere were collected for once a month. Samples were divided into three groups including, swabs materials from fish surface (n=48), viscera (n=46) and lake water (n=60). The samples inoculated on to the Furunculosis Agar and Tryptic Soy Agar including Coomassie Brilliant Blue (C-TSA) and incubated at 25°C for 7 days. Presumptive colonies were examined with biochemical tests for the identification of *A. salmonicida*. Concurrently with bacteriological examination, all of the samples were examined for the presence of vapA gene with Polymerase Chain Reaction (PCR). **Results:** According to bacteriological examination results, *A. salmonicida* were not isolated from any of the samples. As a result of PCR, of 60 water samples 3 (5.0%) were positive for vapA gene, while *A. salmonicida* specific DNA was not detected in the viscere and swab samples. **Conclusion:** It has been determined that infections caused by *A. salmonicida*, especially furunculosis disease, under this light do not constitute a risk in Lake Sazlıdere at the time of the study. However, the information obtained from fishermen and the results of PCR, when evaluated together with chemical contamination in general, indicate that continual investigations should be continued in this area, not within a certain period of time.

Keywords: *Aeromonas salmonicida*; fishes; furunculosis; polymerase chain reaction

ÖZET Amaç: Bu çalışmada balık avcılığının da yapıldığı bir bölge olan Sazlıdere Baraj Gölünde yaşayan tatlı su balık türlerinde ve göl suyunda *Aeromonas salmonicida* varlığının araştırılması amaçlandı. **Gereç ve Yöntemler:** Bu çalışmada, Sazlıdere baraj gölünden Nisan 2014 ve Mart 2015 tarihleri arasında, her ay tek bir gün olmak üzere örnekler toplandı. Örnekler balık yüzeyinden alınan sıvaplar (n=48), iç organlar (n=46) ve göl suyu (n=60) olmak üzere üç grupta toplandı. İnceleme örnekleri Furunculosis Agar ve Coomassie Brilliant Blue ilaveli Triptik Soy Agar (C-TSA)'a ekildi ve 25°C'de 7 gün inkübe edildi. Şüpheli koloniler *A. salmonicida* yönünden biyokimyasal testler yapılarak incelendi. Bakteriyolojk incelemelerle eş zamanlı olarak tüm örnekler vapA spesifik gen bölgesi varlığı yönünden Polimeraz Zincir Reaksiyonu (PZR) ile incelendi. **Bulgular:** Bakteriyolojik inceleme sonuçlarına göre, örneklerin hiç birinden *A. salmonicida* izole edilemedi. PZR sonucunda 60 su örneğinin 3 (%5)'ünde pozitiflik belirlenirken, iç organlar ve sıvap örneklerinde *A. salmonicida* spesifik DNA sı saptanmadı. **Sonuç:** Bu veriler ışığı altında başta furunkuloz hastalığı olmak üzere *A. salmonicida* kaynaklı enfeksiyonların, çalışmanın yapıldığı dönemde Sazlıdere baraj gölünde bir risk oluşturmadığı kanatine varıldı. Ancak balıkçılardan alınan bilgiler ve PZR sonuçları, genel olarak kimyasal kontaminasyonlarla birlikte değerlendirildiğinde bu bölgede belirli bir zaman içerisinde değil sürekli araştırmaların devam etmesi gerekliliğini ortaya koymaktadır.

Anahtar Kelimeler: *Aeromonas salmonicida*; balıklar; furunkuloz; polimeraz zincir reaksiyonu

Aeromonas salmonicida, causes a contagious and lethal infection characterized by the formation of septicemia and deep furuncles called furunculosis in various fish species, especially in trout. Although furunculosis predominantly seen in different fish species all over the world is known as a disease of freshwater fish, recent studies have shown that the disease is also seen in many different fish species living in sea and lakes.[1,2]

Studies conducted in recent years have revealed that *A. salmonicida* has five subspecies: *A. salmonicida* subsp. *salmonicida, A. salmonicida* subsp. *masoucida*, *A. salmonicida* subsp. *achromogenes, A. salmonicida* subsp. *smitha* and *A. salmonicida* subsp. *pectinolytica*. *A. salmonicida* subsp. *salmonicida* causes classical furunculosis termed typical furunculosis. Other subspecies are responsible for atypical furunculosis such as Trout Ulcer Disease, Carp Erythrodermatitis etc. in both salmonids and other fish species.[1,3,4] All subspecies of *A. salmonicida* have the *vapA* gene region. The *vapA* gene region related to the S layer, which is one of the virulence factors of *A. salmonicida.* Because *A. salmonicida,* possesses S layer, has the ability to adhere, enter and to survive within macrophages.[1,5] Gustafson et al. developed the PCR method to detect the *vapA* gene region and identified both typical and atypical *A. salmonicida* species.[5]

The disease has been widely detected in Asia, North America, Scandinavian countries and, especially, in Europe.[1] In Australia, atypical furunculosis has been reported but typical furunculosis has not been reported. *A. salmonicida* isolation has been reported in recent years in New Zealand and South America.[1,6,7] In a polluted river, *A.-salmonicida* was also isolated in Argentina.[8] In Turkey, atypical furunculosis was first detected in cultured sea bass in the Black Sea in 2002.[2] In 2003, *A. salmonicida* was isolated from three trout farming sites in the Western Aegean Region.[9] In a study investigating the aerobic and microaerophilic bacterial flora in the Dardanelles Strait, 59.88% of isolated bacteria were *A. salmonicida* subsp. *achromogenes*.[10] In the marine fish cultivated in the Black Sea, *Photobacterium damselae* subsp. *damselae*, *Aeromonas salmonicida*, *Aeromonas sobria*, *Listonella (Vibrio) angulillaum* and *Staphylococcus epidermidis* were isolated.[11] The studies carried out in Turkey are more focused on cultural fish, especially on rainbow trout.[2,9,11,12] The first and only study carried out in Turkey with wild fish was reported by Onuk et al. The researchers bacteriologically examined the liver, kidneys and spleens of a total of 132 fish from wild fish, farm fish and aquarium fish and isolated *A. salmonicida* from 2 cultivated carp and 1 wild dolphin.[13]

In Turkey, there have been a limited number of studies on cultured trout species in relation to disease, but there are not enough studies on the status of infections in freshwater fish. Due to this lack in the literature, it is aimed to investigate the presence of *A. salmonicida* in freshwater fish species and lake water in the Lake Sazlıdere, a region where fish hunting is also carried out.

MATERIAL AND METHODS

Between April 2014 and March 2015, a total of 165 samples were collected from Lake Sazlıdere. Samples were divided into three groups including, swabs materials from fish surface, viscera (liver and spleen) and lake water. Of the fish species, 27 were *Cyprinus carpio* (Cyprinidae), 13 were *Scardinius erythrophthalmus* (Cypirinidae), 5 were *Perca* sp. (Percidae), 2 were *Gobius* sp. (Gobiidae) and 1 were *Esox lucius* (Esocidae) species. No clinical findings were found in any of the examined fish. Swab samples were collected from 48 fishes, primarily from the gill base and the skin. After swab samples were taken, 70% alcohol solution was sprayed onto the body surface of the 46 fish, the abdominal wall was opened as a semicircle with a sterile lancet.

Swab samples were taken from the surface of all of the 48 fish, liver sample were taken from the same 46 fish and both liver and spleen samples were taken form 11 of them. Lake water samples from different parts of the lake were also collected.

All studies in this article were carried out in line with Guide for the Care and Use of the Laboratory Animals principles and animal rights were protected.

This examination approved by University Animal Experiments Native Ethics Committee.

The date, sample type and number of samples taken are shown in Table 1.

BACTERIOLOGICAL EXAMINATION

Swab samples were transferred into Tryptic Soy Broth (TSB) and incubated at 20°C for 24 hours. One hundred ml of water sample was passed through 0.45 µm filters and the filter was transferred into TSB. The samples were incubated at 20°C for 24 hours. At the end of the incubation period, enriched samples were inoculated to Furunculosis Agar (FA) and Tryptic Soy Agar including Coomassie Brilliant Blue (C-TSA).For the examination of internal organs samples were inoculated directly onto FA and C-TSA. The FA and C-TSA plates were incubated for 72 hours at 25°C for the isolation of *A. salmonicida* and for an additional 4 days for the isolation of atypical *A. salmonicida*. The brown-red colonies on FA and dark blue colonies on C-TSA were considered suspicious.[14] For the identification of the presumptive colonies, oxidase test, catalase test, growth in 6% NaCl, carbohydrate fermentation test, Vibriostatic agent O129 resistance test, motility test, oxidation fermentation test and pigment production test were performed.[15]

MOLECULAR EXAMINATION

Swab samples, internal organs and water samples were analyzed by PCR concurrently with culture studies and the presence of *vapA* gene was investigated in samples. For DNA isolation from swab and water samples, cells were lysed at 95°C for 5 minutes. DNA isolation from tissue samples was performed with the Genomic DNA Extraction Kit (Bioneer).

AccuPower Hotstart PCR Premix (Bioneer) was used for PCR amplification. Twenty ml reaction mixture was prepared from 1 ml of target DNA, 1 ml of Forward primer (AP-1:5'-GGC-TGATCTCTCATCCTCACCC-3'), 1 ml of Reverse primer (AP–2:5'-CAGAGTGAAATCTACCAG-CGGTGC-3'), 17 ml of DNase-RNase free distilled water into the premix and the tubes were inserted into the PCR thermocycler (Axygen). For amplification, the device was programmed for 2 minutes at 95°C, with 30 cycles, 15 seconds at 95°C, 30 seconds at 57°C, 90 seconds at 72°C, and 3 minutes at 72°C. After the gel electrophoresis of the amplified products, the formation of 421 bp specific band was evaluated as positive. *Aeromonas salmonicida* ATCC 33658 and distilled water were used for positive and negative control, respectively.[1]

RESULTS

According to bacteriological isolation results, 65, 22 and 95 suspected colonies in terms of *A. salmonicida* were isolated from 48 skin samples, 57 internal organs and 60 water samples, respectively. After biochemical tests, *A. salmonicida* was not isolated from the samples.

The vapA gene of *A. salmonicida* was found in 3 (5%) of 60 water samples, while *vapA* specific *A. salmonicida* DNA was not detected in 57 internal organs and 48 swabs examined. These positive water samples had been collected in May, December and January. Gel electrophoresis images of positive water samples 8, 45 and 47 are shown in Figure 1.

DISCUSSION

A. salmonicida is one of the most important pathogens of salmonids and is the causative agent of furunculosis. *A. salmonicida* is widespread in all aquatic environments and is the most known member of the family Aeromonadaceae. *A. salmonicida* is an important fish pathogen, which causes serious economic losses especially in aquaculture. *A. salmonicida* has wide geographical distribution in America, Canada, Australia, Japan, Asia and Europe.[16]

It was reported that during the first periods of isolation of *A. salmonicida*, it was only causing infection in salmonids, but in the following years the agent was also isolated in non-salmonid saline and freshwater fishes.[16,17] Other species out of salmonids and can be infected by *A. salmonicida* are minnow, gold fish, carp, perch, bream, roach, dace, chub, tench, pike, bullheads, sculpins and cat

TABLE 1: Distribution of collected samples.

Sampling Date	AMT (°C)	DT (°C)	Sample Type			Sample Number
April 2014	11,5	13	Fish	*Scardinius erythrophthalmus*	L, S	2
				Scardinius erythrophthalmus	L	2
				Gobius sp,	L, S	1
				Gobius sp,	L	1
			Swab			6
			Water			5
May 2014	15,8	16,3	Fish	*Cyprinus carpio*	L	5
			Swab			5
			Water			5
June 2014	20,9	22	Fish	*Perca sp,*	L	1
				Scardinius erythrophthalmus	L, S	1
				Scardinius erythrophthalmus	L	3
			Swab			5
			Water			5
July 2014	24,2	25,1	Fish	*Scardinius erythrophthalmus*	L, S	2
				Cyprinus carpio	L	3
			Swab			5
			Water			5
August 2014	25,5	25,9	Fish	*Cyprinus carpio*	L	5
			Swab			5
			Water			5
September 2014	23,0	22,1	Fish	*Cyprinus carpio*	L	3
			Swab			5
			Water			5
October 2014	18,6	18,8	Fish	*Scardinius erythrophthalmus*	L, S	1
				Perca sp,	L	2
				Cyprinus carpio	L	1
				Cyprinus carpio	L, S	1
			Swab			5
			Water			5
November 2014	14,6	12,3	Water			5
December 2014	11,4	12,4	Fish	*Cyprinus carpio*	L	1
				Cyprinus carpio	L, S	1
				Esox lucius	L	1
			Swab			3
			Water			5
January 2015	8,7	8,2	Fish	*Cyprinus carpio*	L	1
				Perca sp,	L	2
			Swab			3
			Water			5
February 2015	8,0	7,8	Fish	*Cyprinus carpio*	L	2
				Scardinius erythrophthalmus	L, S	1
			Swab			3
			Water			5
March 2015	7,9	7,6	Fish	*Scardinius erythrophthalmus*	L, S	1
				Cyprinus carpio	L	2
			Swab			3
			Water			5

AMT: Average Monthly Temperature; **DT:** Daily Temperature; **L:** Liver; **S:** Spleen.

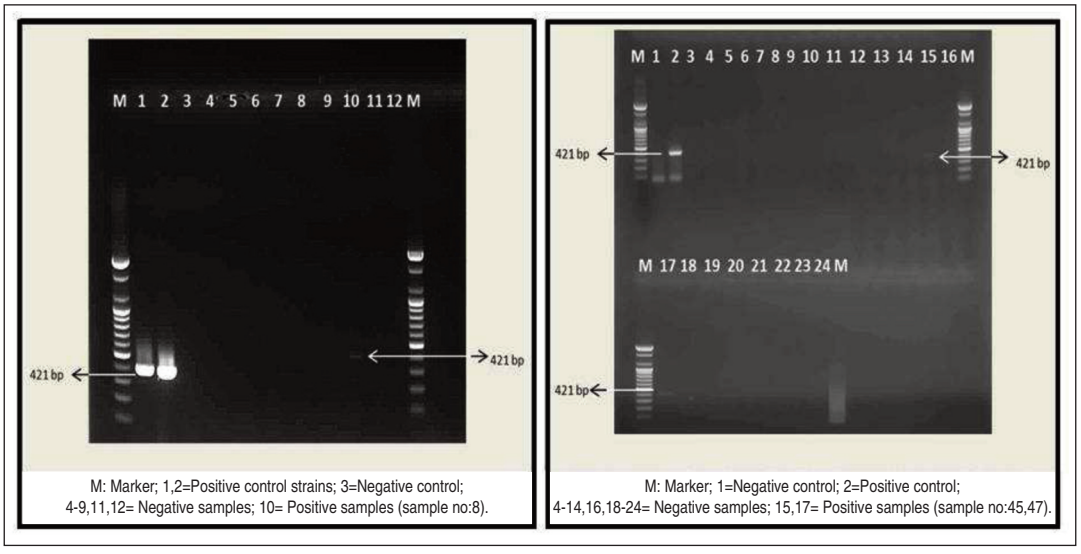

FIGURE 1: Gel electrophoresis images of positive water samples 8, 45 and 47.

fish, wrasse and sea bream.[18] Liver, kidney, spleen, small intestine, gill, mucus, blood, feces, swab and water specimens were selected for *A. salmonicida* isolation. However, in the most of studies liver, kidney and spleen have been examined.[2,5,9-13,16,18-24] In this study, in order to increase the chances of isolation, swab samples were taken from the skin surface of fishes of different species, and also from internal organs such as liver and spleen.

The media used for isolation of *A. salmonicida* are generally TSA, BHI Agar, Blood Agar, Congo Red Agar, Furunculosis Agar and C-TSA. Furunculosis Agar and C-TSA, which are known to be highly selective by many researchers, were used in this study. However, it was observed that during the examination of the water samples, in which the bacterial growth was very intense, too much colony was observed in these two media. This intense growth probably reduced the chance of isolation of the agent. The lack of the isolation from 3 PCR positive water samples, also suggests that *A. salmonicida* colonies have been missed because of the high bacterial growth. In recent years, many types of specific PCR methods have been developed to overcome the difficulties of isolating non-culturable bacteria or bacteria in mixed cultures.[7,16] Bartkova et al. examined 40 fishes from freshwater by real time PCR and culture. In the result of study 65 % of the fishes were detected positive by real time PCR and 30% by the culture. Also, none of the PCR negative samples were found positive by culture.[25] Keeling et al. studied a real-time PCR assay using a molecular beacon to detect the *vapA* gene in *A. salmonicida*.[7] With using kidney tissue of the Japanese fish and Chinook salmon, the researcher found that analytical sensitivity was 2.2×10^4 cfu/g without enrichment and 40 cfu/g with enrichment. This demonstrates the importance of the enrichment step in factor isolation. In this study, all of the samples were enriched in TSB for 24 hours and then analyzed by both culture and PCR.

The optimum temperature of *A. salmonicida* is reported at 18-22°C, but chronic infections can also occur below 13°C. The agent growth stop above 37°C, while slowing below 4°C.[1] It has been reported that seasonal changes are very effective for furunculosis and it has been reported that there is an increase in the furunculosis cases in the late stages of spring and the early stages of summer in which water temperature of 12-15°C.[1,6] It is seen that in the studies in the literature, *A. salmonicida* is isolated even at many different water temperatures, but in most of the studies the isolation rates were concentrated between temperatures of 11-21°C. In this study, samples were collected monthly for 12 months in order to test a wide temperature range and to see possible

changes in the isolation rates at different water temperatures. *A. salmonicida* were not isolated from any of the samples but three positive results were detected in the water samples which were collected in May, December and January. The average water temperatures for those months were 15.8°C, 11.4°C and 8.7°C, respectively. These water temperatures, together with many researchers, were measured by Goldschmidt-Clermont et al., as well.[3] In January the water temperature was below average. During the study, from September onwards, the water level started to fall due to the decrease in precipitation, and even to the point that the fish could not be caught in November. During the following two months, *A. salmonicida* DNA was detected in the water samples. This suggests that *A. salmonicida* may be present in the dam reservoir, although not in intense concentration, and that the decrease in water level may lead to an increase in the detectable concentration of DNA.

Furunculosis may be observed in peracute, acute, subacute-chronic or latent (intestinal) forms.[6] In studies in which *A. salmonicida* has been isolated, clinical signs have been reported, as well as cases where no findings have been reported. The most common clinical findings are pale liver with skin lesions and gills of fish and muscle lesion, gill hemorrhage, spleen and kidney swelling.[2,3,9,17,26-28] Mooney et al. Akşit and Kum**,** Beaz-Hidalgo et al., Kim et al. Onuk et al. have not found any clinical findings in fishesfrom which *A. salmonicida* were examined.[4,13,16,23,24] In this study 46 fishes were examined and no clinical findings were observed in any of them. As a result of both culture and PCR, *A. salmonicida* was not detected from fish samples. Clinical findings were consistent with laboratory findings. However, during the collection of the samples, some fishermen indicated that they have observed some skin lesions in some fishes. Those daily observations of the fishermen and PCR positive results of 3 water samples, reveals the presence of *A. salmonicida* even with a low rate.

In this study, *A. salmonicida* were not isolated by culture and not detected by PCR from fish samples. However, *A. salmonicida* DNA were detected in 3 of water samples. According to those laboratory findings, the authors can suggest that infections caused by *A. salmonicida*, especially furunculosis, do not constitute a risk in Lake Sazlıdere at the time of the study. However, information from fishermen and PCR results, once evaluated together with chemical contamination in the lake in general, once again reveals the necessity of continuing the ongoing investigations, not within a certain period of time.

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Conflict of Interest

No conflicts of interest between the authors and / or family members of the scientific and medical committee members or members of the potential conflicts of interest, counseling, expertise, working conditions, share holding and similar situations in any firm.

Authorship Contributions

Idea / Concept: *Ayşe Sezgin, Arzu Funda Bağcıgil;* ***Design:*** *Ayşe Sezgin, Baran Çelik, Beren Başaran Kahraman, Arzu Funda Bağcıgil;* ***Inspection / Consultancy:****, Elif İlkay Armutak, Arzu Funda Bağcıgil;* ***Data Collection and/or Processing:*** *Ayşe Sezgin, Baran Çelik, Beren Başaran Kahraman, Elif İlkay Armutak, Arzu Funda Bağcıgil;* ***Analysis and/or Comment:*** *Ayşe Sezgin, Baran Çelik, Beren Başaran Kahraman, Arzu Funda Bağcıgil;* ***Resource Screen:*** *Ayşe Sezgin, Baran Çelik, Beren Başaran Kahraman;* ***Maternal Writing:*** *Ayşe Sezgin, Baran Çelik, Beren Başaran Kahraman, Elif İlkay Armutak, Arzu Funda Bağcıgil;* ***Critical Review:*** *Elif İlkay Armutak, Arzu Funda Bağcıgil.*

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