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Investigation of diseases caused by *Aeromonas media* in rainbow trout (*Oncorhynchus mykiss)* in commercial fish farms using MALDI-TOF and specification of antibiotic sensitivity profiles of the agent

Investigación de enfermedades causadas por *Aeromonas media* en truchas arcoíris (*Oncorhynchus mykiss*) en piscifactorías comerciales utilizando MALDI-TOF y especificación de perfiles de sensibilidad antibiótica del agente

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ABSTRACT

Rainbow Trout (RT) *Oncorhynchus mykiss* Walbaum,1792 the first fish species to be grown intensively in Turkey and the most commonly grown species in dams and lakes today. *Aeromonas* spp. are found in ecosystems such as freshwater and coastal waters and cause disease in fish. In RT causes death that is characterized by internal organ hemorrhages, such as the spleen, kidney and liver and body surface, ulcerations and congestion. The present study aimed to detect *Aeromonas media* bacteria in fish samples collected from the RT farms in the South Eastern Anatolia Region of Turkey through bacteriological culturing and MALDI-TOF. Also, it was purposed to determine the antibiotic susceptibility of the agent. Antibiotic susceptibility testing was performed using the Kirby–Baüer disk diffusion method. Separate visits were made to Trout farming facilities in Diyarbakır, Adıyaman, Şanlıurfa, and Batman in January and February 2021, and 30 fish with an average live weight of 200–250 grams (g) were collected from each facility. A total of 1,200 samples were taken from 40 Trout farms. It was detected the causative agent in the liver, kidney, and tissue samples of fish from 28 of the 40 farms. The sensitivity of Enrofloxacin (10 microgram -µg-), Florfenicol (30 µg), Neomycin (10 µg), Amoxicillin (10 µg), Oxytetracycline (30 µg), Erythromycin (10 µg), Gentamycin (10 µg) and Streptomycin (10 µg) were defined at chancing ratios. In conclusion, these bacteria were detected in local farms. These farms must implement measures to minimize stress factors affecting the fish and avoid overstocking. The best methods of protection against infection include avoiding overstocking, low oxygen levels, and unskilled labor.

Key words: *Aeromonas media*; MALDI-TOF; *Oncorhynchus mykiss*

RESUMEN

La trucha arcoíris (TA) *Oncorhynchus mykiss* Walbaum,1792 es la primera especie de pez que se cultiva de forma intensiva en Turquía y la especie que se cultiva más comúnmente en presas y lagos en la actualidad. *Aeromonas* spp. se encuentran en ecosistemas tales como agua dulce y aguas costeras y causan enfermedades en los peces. En la TA provoca la muerte que se caracteriza por hemorragias en órganos internos, como el bazo, riñón e hígado y superficie corporal, ulceraciones y congestión. El presente estudio tuvo como objetivo detectar la bacteria *Aeromonas media* en muestras de peces recolectados de las granjas de TA en la región de Anatolia del sureste de Turquía, a través del cultivo bacteriológico y empleo de MALDI-TOF. Asimismo, se propuso determinar la susceptibilidad antibiótica del agente. Las pruebas de susceptibilidad a los antibióticos se realizaron utilizando el método de difusión en disco de Kirby-Baüer. Se realizaron visitas separadas a las instalaciones de cultivo de T en Diyarbakır, Adıyaman, Şanlıurfa y Batman, en enero y febrero de 2021, y se recolectaron 30 peces con un peso vivo promedio de 200 a 250 gramos (g) en cada instalación. Se tomaron un total de 1.200 muestras de 40 criaderos de T. Se detectó el agente causal en muestras de hígado, riñón y tejido de peces en 28 de las 40 granjas. La sensibilidad de enrofloxacina (10 microgramo -µg-), florfenicol (30 µg), neomicina (10 µg), amoxicilina (10 µg), oxitetraciclina (30 µg), eritromicina (10 µg), gentamicina (10 µg) y estreptomicina (10 µg) se definieron en proporciones de probabilidad. En conclusión, estas bacterias fueron detectadas en granjas locales. Estas granjas deben implementar medidas para : minimizar los factores de estrés que afectan a los peces y evitar el exceso de población. Los mejores métodos para protegerse contra la infección incluyen : evitar el exceso de existencias, los bajos niveles de oxígeno y la mano de obra poco calificada.

Palabras clave: *Aeromonas media*; MALDI-TOF; *Oncorhynchus mykiss*

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INTRODUCTION

Health authorities worldwide emphasize the health benefits of fish and recommend fish consumption at least two times per week (wk) as part of a healthy diet. While the World Health Organization (WHO) states that a daily intake of 250–300 miligrams (mg) of Eicosapentaenoic acid + Docosapentaenoic acid is beneficial, the American Heart Association states that it is necessary to consume 340 grames (g) of fish per wk [27].

Global aquaculture production in 2020 reached a record 122.6 Mil Ton, including 87.5 Mil Ton of aquatic animals worth USD 264.8 Bill and 35.1 Mil Ton of algae worth USD 16.5 billion. Around 54.4 Mil Ton were farmed in inland waters and 68.1 Mil Ton came from marine and coastal aquaculture [12]. Recently, with the great efforts of aquaculture farmers, Turkey has become one of the major producers of aquaculture both in Europe and globally**.** According to TURKSTAT data, aquaculture production increased by 12.9% in 2020 compared to 2019, with Trout (T) being the most important fish species [26].

With the increase in aquaculture production, bacterial diseases are among the leading causes of losses due to fish diseases under intensive aquaculture conditions. The most common infective causative agents include bacteria, such as *Aeromonas* spp. *Renibacterium salmoninarum*, *Flavobacterium* spp., *Yersinia ruckeri*, and *Vibrio* spp. Among these bacterial fish pathogens, the genus *Aeromonas* is notable for having a large number of species [6].

Commercial Rainbow Trout (RT) farming throughout Turkey is carried out in different water temperatures, stream types (e.g., dams, spring water, or rivers), and breeding conditions. *Aeromonas* spp. an opportunistic pathogen, causes infections in cases of sudden changes in water temperature and increased stress factors. Although motile *Aeromonas* spp., the causative agent of motile *Aeromonas* septicemia is not pathogenic to fish, it is responsible for various infectious complications that have resulted in mortality and outbreaks in humans [10]. Clinical signs of fishes infected by these pathogens include hemorrhage and ulcer formation on the skin, darkening of the skin, internal hemorrhages, enlargement of the spleen, exophthalmos of one or both eyes, fin rot, fluid accumulation, swimming disorders, and loss of appetite, with high mortality rates reported [28].

In this study, samples were collected from different T farming facilities in Diyarbakır, Şanlıurfa, Adıyaman, and Batman Provinces to investigate *A. media*. These samples were transported to the laboratory through cold chain. Following the macroscopic examination, isolation and identification were performed using culture and MALDI-TOF detection, respectively and antibiotic susceptibility was determined. To ease fast and reliable identification of aquatic and piscine bacteria, matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) [14] has lately been presented as a useful method instrument [25]. It allows high throughput, sensitive and specific applications in clinical diagnostics and profiling of bacteria to the genus, species and even strain-level [24]. The main purpose of the study was to reduce losses by diagnosing and treating fish diseases correctly.

MATERIALS AND METHODS

Samples were collected from 40 different commercial RT facilities in Diyarbakır, Adıyaman, Şanlıurfa, and Batman Provinces in the South Eastern Anatolia Region. Thirty fish with an average live weight of 200–250 g; some clinical signs such as fin rot, lesions and ulcers on the skin, skin darkening, and exophthalmos were considered and unresponsive fish to feeding and immobile in a certain part of the pool or floating on the water surface were sampled from each farm.

In total, 1,200 samples were taken to the laboratory for macroscopic examination before being dissected for internal organs evuluation. Liver, kidney, and tissue samples obtained from dissection were inoculated into the medium to identify bacteria. Following the macroscopic findings, necropsies of the fish delivered to the laboratory in the cold chain were performed and samples were taken with sterile swabs. Swab samples were first transferred to MacConkey Agar for self-enrichment. The MacConkey Agar (MAC) medium was used for bacteria isolation seeded plates were incubated for 24 hours (h) at 28°C, and the dominant uniform bacterial colonies were purified by streaking three times onto MAC plates [2]. Then, the causative agents were identified using MALDI-TOF mass spectrometry by Maldi Biotyper (Bruker, Daltonics, Germany) based on Gram staining and culture growth profiles (FIG. 1).

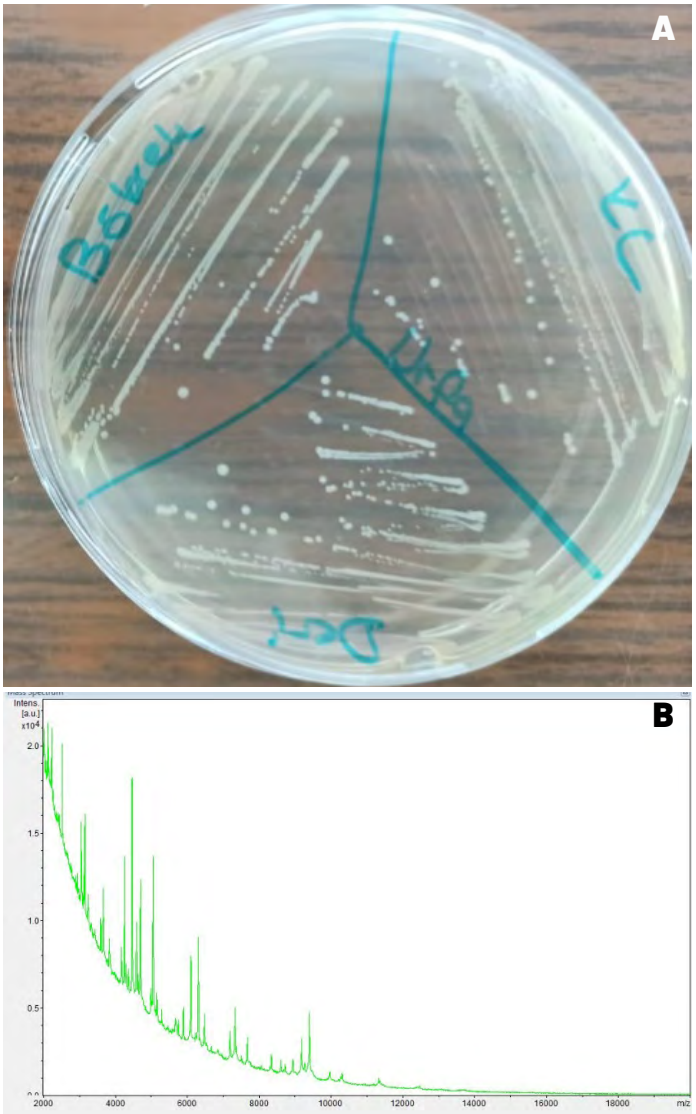

FIGURE 1. (A) Colony morphology and (B) Spectrum images of MALDİ-TOF of *Aeromonas media*

Antibiotic susceptibility testing was performed using the Kirby–Baüer disk diffusion method, Müeller–Hinton medium, and the method described by Bauer et al. [4]. and evaluated according to the procedures reported by the Clinical and Laboratory Standards Institute (CLSI) [8], Ruangpan and Tendencia [22] and Becton, Dickinson and Company [7].

RESULTS AND DISCUSSION

In the present study, *A. media* were culture isolated and MALDI-TOF identified from fish with suspected disease from 40 different RT (*Oncorhynchus mykiss*) In 28 out of 40 facilities, 1,200 (30 fish/40 farm) samples with stagnation, anorexia, exophthalmos, swimming disorders, loss of appetite, darkening and color changes of the skin, and hemorrhages on the base of fins were determined, and the causative agent were determined. The causative agent was identified in the liver, kidneys, and tissues of 840 samples. The clinical signs in infected fish from which tissue samples were taken for bacterial isolation and identification included kidney hemorrhage, congestion and pallor in the liver, erythema and hemorrhage on the gill lamellae, yellow-colored fluid accumulation in the intestines, and tail and fin rot (FIG. 2).

It was determined that 0-100% of the T farms in the South Eastern Anatolia Region were contaminated with *A. media*, and the agent develops varying levels of resistance to antimicrobial agents. The incubation period of the disease varies depending on environmental conditions, care and feeding conditions, age, species and immunity, accompanying infections, virulence, number, and route of entry of microorganisms. A larger number of bacteria were isolated from T farms with low water flow, overpopulation, and turbid pool water. Antibiogram test results revealed sensitivity to Enrofloxacine, Florfenicol, and Gentamycine (TABLE 1).

Global population growth and nutritional issues emphasize the value of aquaculture products (AP) as a high-protein source. To meet a significant portion of the protein demand, it is crucial to maintain the sustainability of aquatic foods and increase the production of AP in inland and outer waters. The continued expansion of aquaculture production is regarded as a key strategy for ensuring global food and nutritional safety and closing the "fish gap," which is the disparity between seafood supply and demand [13].

TABLE 1
Antimicrobial susceptibility of *Aeromonas media*

Antimicrobial agent	Isolate 1	Isolate 2	Isolate 3
Enrofloxacin (10 µg)	S	S	S
Florfenicol (30 µg)	S	S	S
Neomycin (10 µg)	R	R	R
Amoxicillin (10 µg)	R	R	R
Oxytetracycline (30 µg)	R	R	R
Erythromycin (10 µg)	R	R	R
Gentamycin (10 µg)	S	S	S
Streptomycin (10 µg)	R	R	R

µg: microgram. S: Susceptible, R: Resistant

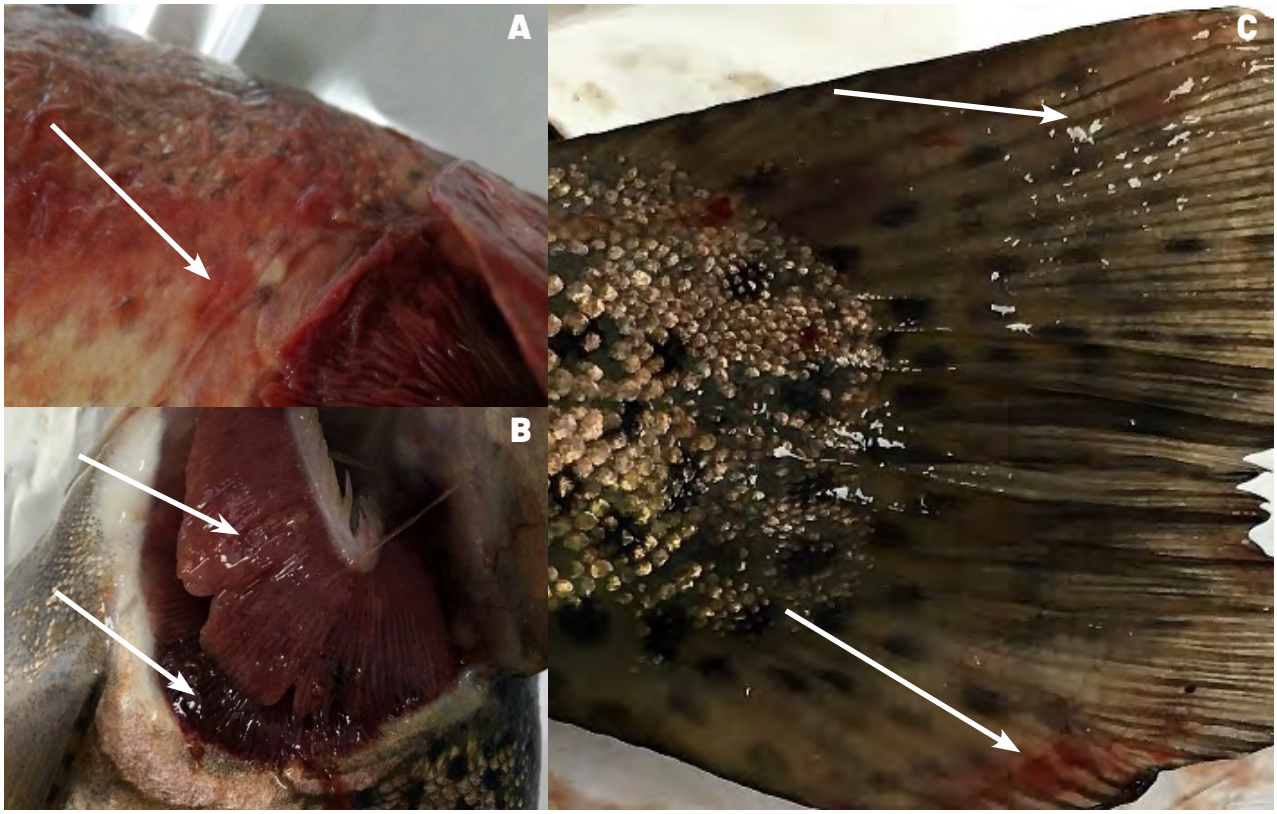

FIGURE 2. (A) White arrow: Red and petechial hemorrhages on the skin. (B) and (C) White arrows: Erythema and hemorrhages on the gill and fin

Diseases of farm-raised fish have become more common with the development of aquaculture, and facilities have begun to take hygienic and protective measures to control diseases. Excessive use of chemicals and antibiotics may result in higher levels of their residues in fish meat and nature and could also lead to fish immune system suppression weakening immunity against pathogens [9]. Accordingly, proper bacterial detection and antibiotic use are required. The genus *Aeromonas* is responsible for acute, subacute, chronic, or latent infections in fish, birds, mollusks, and humans [3]. It results in hemorrhagic septicemia in brown T (*Salmo trutta*) goldfish (*Carassius auratus*), eel (*Anguilla anguilla*), carp (Cyprinus carpio), sweet fish (*Plecoglossus altivelis*), RT (*Oncorhynchus mykiss*) chinook salmon (*Oncorhynchus tshawytscha*), *tilapia,* (*Oreochromis niloticus)* and other farm-raised fish species [5].

Allen *et al.* [1] first reported *A. media* isolated from fish pools and water sources. *A. media* is a common pathogen found in lakes and rivers and acts as an intracellular pathogen in fish [13, 17]. In recent years, *A. media* has been widely reported. The main findings include fin rot, hyperemia, abdominal distension and redness [15], and ulcer formation on the skin [16]. These infectious causative agents of fish are most prevalent in polluted waters [5]. Bacterial agents in fish greatly affect fish health and the aquaculture economy, leading to financial losses if treatment is not initiated early. Acute infections cause a high mortality rate in fish, whereas chronic infections have a low mortality rate [23]. It was determined that 0-100% of T Farms in the South Eastern Anatolia Region were contaminated with *A. media*, and the causative pathogen exhibits varying levels of resistance to antimicrobial agents.

In present study, the findings on infected fish were similar to other studies. Austin and Austin [3] reported redness and inflammation of the fins, widespread bleeding in the anus and skin, swelling of the eyes and abdomen (ascites), intestinal hemorrhages, and bleeding and swelling of the tissues such as the spleen and kidneys. In present study, similar findings such as bleeding in the kidney, congestion and pallor in the liver, erythema and hemorrhage on the gill lamellae, accumulation of yellow-colored fluid in the intestines, tail and fin rot, as well as fin redness were found (FIG. 2).

The high stock density of fish of different sizes, poor water quality, and sudden changes in water temperature can cause *A. media* infections in fish. Infections due to these bacteria, negatively affect the fish farming economy, with high mortality in cases of dense fish capacity [18]. The reasons mentioned above could explain why pathogenic agents were detected in 28 out of 40 facilities in the present study. The cleaning conditions of the pools were extremely poor in the aquaculture facilities where the pathogenic agent was detected. Poor hygiene and cleaning may have led to poor water quality and the rapid spread of infection. A higher number of bacteria were isolated from facilities where the pool water was turbid and polluted.

Previous studies have also shown that factors such as care, nutrition, changes in the physical and chemical structure of water, decreased oxygen content, and water pollution influence the emergence and spread of the disease [11]. Disease recurrence can be prevented by improving water conditions, avoiding excessive stocking density, and taking protection and control measures.Treatments that are not specific to the pathogenic agent do not cure the diseases, and financial losses and adverse consequences occur as a result of inappropriate drug and chemical use [20]. Another issue is that antibiotics are harmful to the environment, and inappropriate use may lead to drug resistance in fish [21]. In the literature, increased bacterial resistance to drugs in aquaculture farms has been reported [15, 24]. Accordingly, testing antibiotic susceptibility is critical to providing guidance for treatment. In the present study, antibiogram test results revealed Enrofloxacine (10 µg), Florfenicol (30 µg) and Gentamycine (10 µg) susceptibility. The results of antibiotic susceptibility in the study of Lü *et al.* [19]. on *A. media* is in line with the present findings.

CONCLUSION

Pathogen positive was found in 840 of the samples examined. Among the tested antibiotics, Enrofloxacin (10 µg), Florfenicol (30 µg), Gentamycin (10 µg) were found to be sensitive to the pathogen. Appropriate care and feeding conditions, improved water quality, reduced stock intensity, and the removal of dead fish can reduce disease emergence and result in faster treatment results. Attention should be paid to cleaning and hygiene. With regular pool cleaning, both fecal and feed wastes should be removed; water circulation should be ensured, and fish scoops, buckets, and nets should be cleaned. Furthermore, water temperature and oxygen levels should be monitored regularly.

Conflicts of Interest

The authors declare that they have no conflicts of interest in the research.

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