

Assessment of the presence of furunculosis in cultured *Oncorhynchus mykiss*

Key words

Aeromonas salmonicida;
antibiotic;
fish farms;
furunculosis;
ulcer

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Abstract: Furunculosis is a significant and pervasive disease affecting fresh and sea-water fish globally. The disease is caused by a specific etiological agent, *Aeromonas salmonicida*, which leads to the formation of furuncles, or ulcerative muscle lesions, and other cutaneous manifestations such as red patches and skin discoloration. A total of 15 trout farms were visited, and 300 samples were taken from 60 individuals with an average live weight of 100-180 grams. Subsequently, the bacteria were identified through culture and MALDI-TOF. The severity of pathological symptoms correlates with the virulence of the disease. The agent has been detected in fish at temperatures between 15°C and 19°C. 40 % of the fish exhibited exophthalmus, 38.7 % displayed sluggish swimming, 38.3 % exhibited lethargic swimming, and 46.67 % exhibited loss of appetite. The prevalence of fish exhibiting pallor of the gills was 45 %. Furthermore, 46.7 % of the fish exhibited haemorrhages in the skin and fins. Ulcer formation on the skin was observed in 36.7 % of the fish. An internal examination revealed the presence of bloody fluid accumulation in the intestines of the fish at a rate of 23.7 %. Furthermore, 29 % of the fish exhibited splenomegaly, while 23.3 % displayed hepatomegaly and pigmentation changes. The aforementioned symptoms were observed with greater frequency on farms belonging to Group A. An examination of the antimicrobial susceptibility of *A. salmonicida* revealed that florfenicol (30 µg), enrofloxacin (10 µg), amikacin (10 µg) and gentamycin (10 µg) demonstrated sensitivity.

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Introduction

The aquaculture industry is experiencing significant financial losses on a global scale due to the prevalence of infectious diseases, including bacterial, viral, fungal, and parasitic pathogens (1). It is estimated that approximately 35 % of the diseases that affect the tissues of juvenile and adult fish are caused by bacterial pathogens, rendering them more susceptible to infection (2). In freshwater fish, furunculosis is caused by the bacterium *Aeromonas salmonicida*. *A. salmonicida* is a Gram-negative, facultatively anaerobic and non-motile bacterium (3). It is not only the causative agent of furunculosis, but also causes septic lesions leading to a high mortality rate (4). Furthermore, there is a growing body of evidence indicating that mesophilic strains of the bacterium have the potential to infect humans, as evidenced by reports of human infections (5). The primary means of transmitting *A. salmonicida* is through close contact with

an infected fish or through the gills, which are often damaged by other pre-existing infections (6). Following entry, the organism is able to spread systemically from the gills due to the presence of abundant blood capillaries in this organ. Nevertheless, the most common routes of infection are the skin and digestive tract (7). Moreover, the composition of mucus in the skin or intestine is a potential contributing factor in the development of infection and subsequent bacterial adhesion (8). The disease presents a range of clinical manifestations that are contingent upon the age, health and species of the fish, along with external factors such as environmental temperature (9). The more acute form of furunculosis is most commonly observed in younger fish, while the peracute form is most frequently seen in fingerling fish. The disease is typically observed in young animals approximately one year of age, resulting in

Table 1: The table provides an overview of the available information on fish farms and samples

	Temperature of the aquaculture water	Level of oxygen in the water	Number of farms sampled	Number of fish collected from farms	Total number of samples	Average live weight of fish
A Farms	15±1°C	8.5 mg/L	4	20	80	100-180gr
B Farms	17±1°C	8.6 mg/L	6	20	120	100-180gr
C Farms	19±1°C	8.4 mg/L	5	20	100	100-180gr

generalized bacterial sepsis and a high mortality rate within two to three days. A chronic form has been documented in mature fish, characterized by the presence of distinctive boils in muscles (10). *O. mykiss* is the most widely cultured fish species in Turkey. However, there is a notable dearth of information regarding furunculosis outbreaks in farmed *O. mykiss* in the country. Therefore, the objective of this study was to ascertain the prevalence and aetiology of furunculosis in trout.

In order to achieve this objective, pathological findings will be identified in the samples collected by conducting extensive visits to numerous fish farms. The presence of *A. salmonicida* will be confirmed through the utilisation of culture and MALDI-TOF. Following the detection of the bacteria, the sensitivity to antibiotics will also be determined.

Materials and methods

Fish Samples

All of the sampled enterprises are engaged in the practice of net cage farming. The selection of farms for investigation was conducted randomly. A total of fifteen fish farms situated in the south-eastern region of Turkey were subjected to a comprehensive examination. A total of twenty fish samples were obtained from individuals exhibiting signs of illness, with an average weight of 100-180 grams. The temperature of the water in the fish farms from which the samples were obtained exhibits fluctuations between 15 and 19°C. Additionally, the oxygen level was recorded as 8.4-8.6 mg/L (Table 1).

The feeds utilised in fish farming operations are sourced from a range of commercial entities, and their nutritional profiles exhibit considerable variation (Table 2).

Clinical investigation

The behaviour of fish in the pools was monitored, and samples were taken from fish exhibiting a range of abnormal behaviours and conditions. The observations included the presence of fish floating on the water's surface, fish with shed scales, fish deemed unappetising, fish motionless at the bottom of the pool, fish with abnormal swimming behaviours, fish not eating, fish exhibiting signs of weakness

and sluggishness, fish with respiratory distress, and fish with lesions on their fins and skin. The necropsy method was conducted in two distinct phases: an external examination and an internal examination. In the external examination, a comprehensive analysis was conducted on the skin, mouth, eyes, gills, anus, operculum and fins, with the findings meticulously recorded. Samples were obtained from areas exhibiting aberrant characteristics, including erosion, bleeding, lesion formation, mucus accumulation, the formation of any mass or ulceration on the skin (Fig 2, 3). A clinical examination was conducted on the fish, and the external clinical signs observed included haemorrhages on the mouth, fins and eyes, skin and gill discolouration, bleeding at the base of fins, inside and around the mouth, and exophthalmia. The aforementioned data were recorded in accordance with the methodology delineated by Schaperclaus (11). The internal (postmortem) examination was conducted in adherence to the procedures outlined by Austin (12). Following necropsy, a comprehensive examination of the internal organs was conducted, encompassing the liver, gallbladder, pancreas, kidney, spleen, gastrointestinal tract, swim bladder, gonads, and heart. The dimensions and pigmentation of the liver were also assessed, as were any abnormalities observed in the spleen, including indications of growth and degeneration, which were duly recorded. The samples were subjected to microbiological analysis. Samples were obtained from the liver, spleen, and skin for microbiological examination. Swab samples were taken from ulcerated lesions on the skin and fins, the liver, the spleen, the kidney, the gills and the intestines. The MacConkey Agar (MAC) medium was employed for the isolation of bacteria for a period of 24 hours at a temperature of 28°C. The dominant, uniform bacterial colonies were then purified through streaking three times onto MAC plates. Thereafter, the causative agents were identified through the utilisation of MALDI-TOF mass spectrometry, specifically the Maldi Biotyper (Bruker, USA), which was based on the results of Gram staining and the profiles of the cultures (Fig 1). The Kirby–Bauer disk diffusion method, Mueller–Hinton medium, and the methodology outlined by Bauer (13) were employed in the conduct of antibiotic susceptibility testing. The results were evaluated in accordance with the procedures recommended by the Ruangpan and Tendencia, Clinical and Laboratory Standards Institute, and Becton, Dickinson and Company (14, 15, 16).

Table 2 Presents the feeds and nutritional contents utilised in fish farming operations

	Feed 1 (A farms)	Feed 2 (B Farms)	Feed 3 (C farms)
Protein Content	45.42 %	45.3 %	45.0 %
Crude fat	20.07 %	22.2 %	23.5 %
Cellulose	1.84 %	1.6 %	1.1 %
Crude Ash	9.21 %	5.7 %	8.5 %
Phosphorus	1.72 %	0.75 %	0.8 %
Calcium	2.30 %	2.00 %	2.10 %
Lysin	2.47 %	2.40 %	2.40 %
Metionin	1.13 %	1.10 %	1.10 %
Vitamine A	3500 UI/kg	3500 UI/kg	3500 UI/kg
Vitamine D3	850 UI/kg	825 UI/kg	800 UI/kg
Vitamin E	155 mg/kg	155 mg/kg	155 mg/kg
Vitamin C	110 mg/kg	110 mg/kg	110 mg/kg

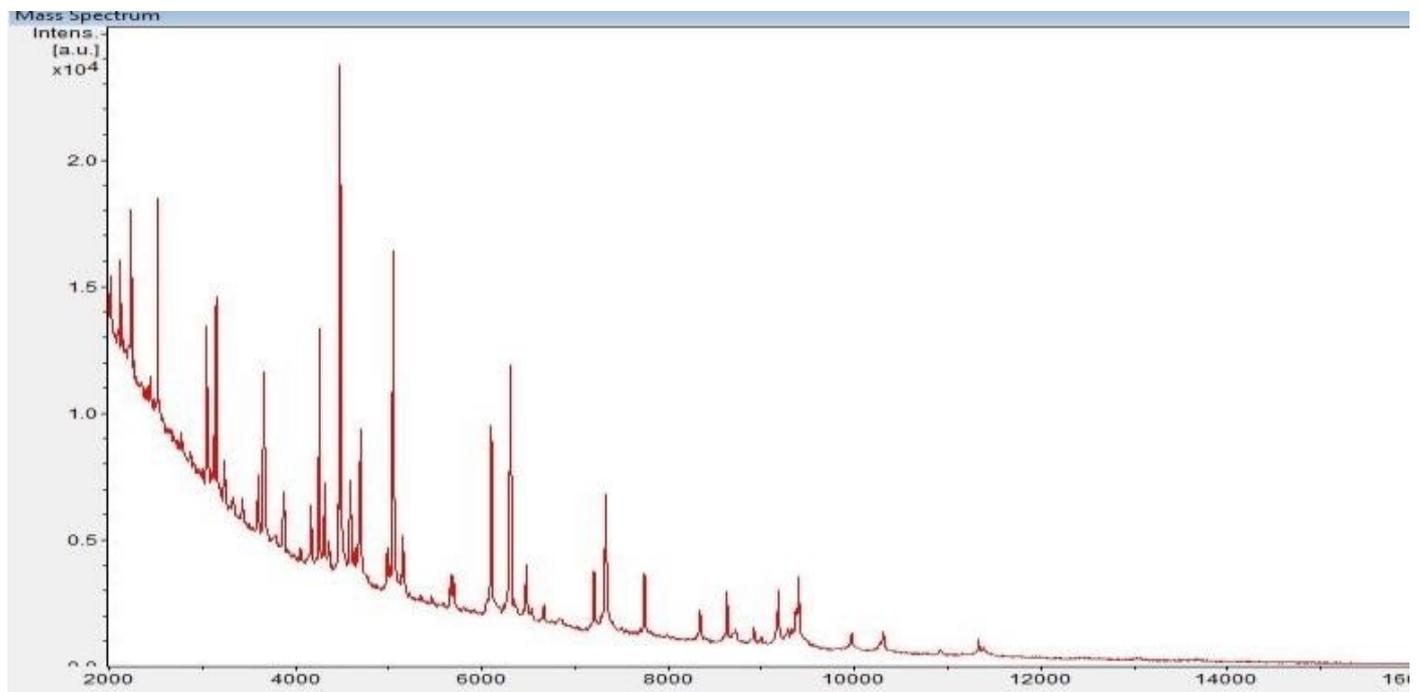


Figure 1: MALDI-TOF spectrum images (*A. salmonicida*)

Results

In the general examination of the fish in the study, the following observations were made: 40 % exhibited exophthalmus, 38.7 % displayed sluggish swimming, 38.3 % exhibited lethargic swimming, and 46.67 % exhibited loss of appetite.

The percentage of fish exhibiting paling of gills was 45 %. Additionally, 46.7 % of the fish displayed hemorrhages in the skin and fins. The gills of 43.3 % of the fish were observed to open and close rapidly. Ulcer formation on the skin was noted in 36.7 % of the fish (Fig 4). The internal examination revealed the presence of bloody fluid accumulation in the



Figure 2: The progressive formation of furunculosis on fish skin

intestines of the fish at a rate of 23.7 %. Additionally, spleen enlargement was observed in 29 % of the fish, while liver enlargement and darkening were noted in 23.3 % of the fish and anus protrusion was observed in 11 % of the fish (Fig 4). The mortality rate of fish exhibiting symptoms of disease in aquaculture facilities exhibits considerable variability, with reported rates ranging from 20 % to 100 %.

The fish farms were categorised into three distinct groups in accordance with the specifications outlined in the Material method. Upon evaluation of the farms in comparison to one another, it was observed that the highest prevalence of exophthalmos was observed in Group C, with a rate of 60 %. The lowest observed rate was that of Group A, which was recorded at 25 %. The findings in Group C, which exhibited the highest rate, included sluggish swimming, lethargy,

Table 3. The aforementioned classification system (S for susceptible and R for resistant) has 186 been employed to ascertain susceptibility to antimicrobial agents in *A. salmonicida*

Antimicrobial agent	Paper content (µg/piece)	Isolate 1	Isolate 2	Isolate 3
Enrofloxacin	10	S	S	S
Florfenicol	30	S	S	S
Neomycin	10	R	R	R
Amikacin	10	S	S	S
Oxytetracycline	30	S	S	S
Erythromycin	15	R	R	R
Gentamycin	10	S	S	S
Streptomycin	10	R	R	R

loss of appetite, pallor of gills, hemorrhages in the skin and fins, gills that opened and closed quickly, ulcer formation on the skin, and a combination of these (Fig 4).

The incidence of spleen enlargement, liver enlargement and darkening, and accumulation of bloody fluid in the abdominal cavity was found to be highest in Group C farms, while the lowest rates were observed in group A farms. Protrusion of the anus was also noted, with the lowest rate occurring in Group A and the highest in Group C (Fig 4).

An examination of the antimicrobial susceptibility of *A. salmonicida* revealed that, florfenicol 180 (30 µg), enrofloxacin (10 µg), amikacin (10 µg) and gentamycin (10 µg) were found to be 181 sensitive, while neomycin (10 µg), oxytetracycline (30 µg), erythromycin (15µg) and 182 streptomycin (10 µg) were found to be resistant (Table 3).

Discussion

It has been demonstrated that all trout farms are infected with *A. salmonicida*, which exhibits varying degrees of intrinsic to antimicrobial agents. The duration of the disease's incubation period is contingent upon a number of factors, including environmental conditions, maintenance and feeding practices, host immunity, microbial virulence, the number of microorganisms involved, and the route of entry. The most striking finding is the correlation between water temperature and the prevalence of *A. salmonicida* and pathological findings. Samples from enterprises with high water temperatures demonstrated a higher incidence of these factors (17).

The observed lesions and clinical manifestations indicate that the juvenile *O. mykiss* is afflicted with a naturally



Figure 3: illustrates the presence of a hemorrhagic focus and bleeding in the fin (a), further hemorrhagic focus and wound formation around the eye (b), wound formation around the mouth (c), ulcer formation and necrotic foci on the skin (d)

occurring infection of the fish by *A. salmonicida*, which presents as a hyperacute form of the disease. Characteristic of this form is hyperpigmentation of the skin, dyspnoea and an elevated mortality rate among the fish. Additionally, the presence of numerous haemorrhages in several organs, in conjunction with the severe ulcerative dermatitis, is a distinctive feature of acute furunculosis (6). The haemorrhagic septicaemia evident in specimens of fish is indicative of a hyperacute form of furunculosis (4). *A. salmonicida* infection presents in three distinct clinical forms. The hyperacute form is the most prevalent in younger individuals, whereas the chronic form is the most common in adults. The presence of pre-existing dermal lesions, such as skin abrasions, has been identified as a predisposing factor for the development of ulcerative dermatitis in fish (18).

Dermal abrasions can be caused by a variety of mechanisms, primarily poor handling of animals and the coexistence of other infectious (viral or bacterial) or parasitic diseases, which were excluded from this study. Moreover, the environment is a crucial factor in the emergence and progression of fish diseases. The species *O. mykiss* is

highly sensitive to temperature fluctuations, with deviations outside the physiological range potentially leading to heat stress, increased morbidity and mortality. Indeed, the study demonstrated that the virulence of the disease increased in line with the temperature difference between the enterprises. A further significant finding is consistent with the conclusions of Okoli (19) who proposed that the contamination of wild fish stocks by *Aeromonas spp.* from contact with infected fish is a key risk factor in the outbreak of furunculosis. Documentation indicates that horizontal transmission of infection from infected fish to uninfected stocks may occur through physical contact or the spillage of bacteria into the water. The mechanism of horizontal infection transmission of atypical species of *Aeromonas spp.* appears to be similar in this regard (20).

Despite the optimal growth temperature of *A. salmonicida* being reported as 18-22°C, chronic infections can also occur below 13°C. The reproduction of the agent is inhibited below 4°C and ceases above 37°C (21). It has been demonstrated that seasonal changes exert a significant influence on the occurrence of furunculosis. There have been

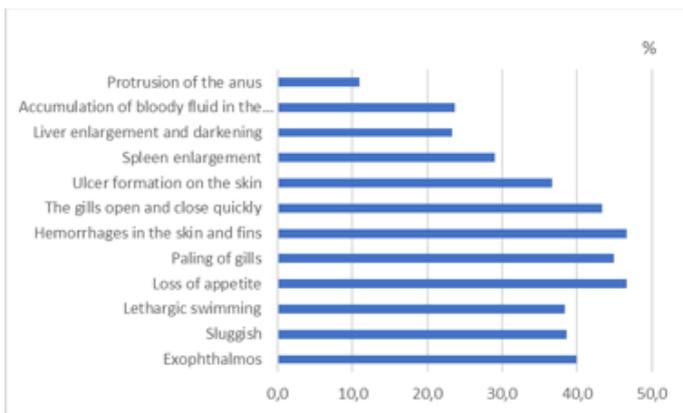
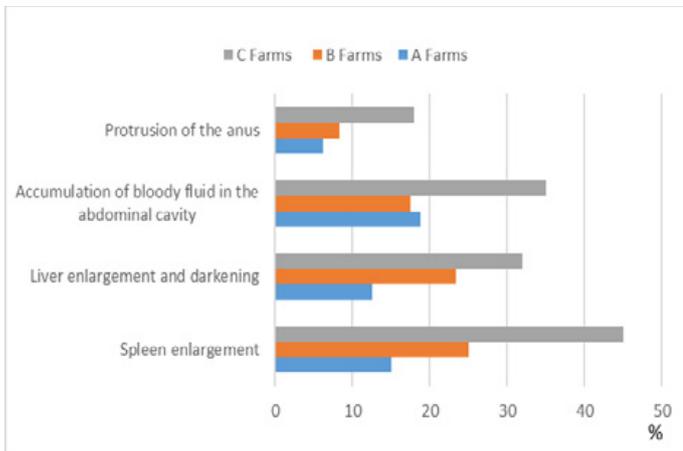
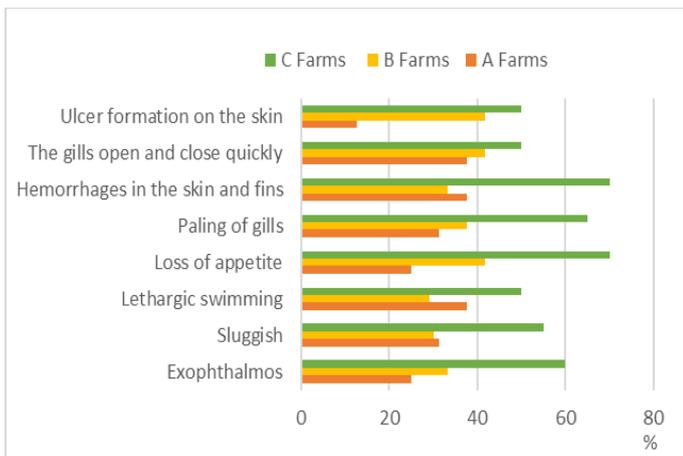


Figure 4: The graph illustrates the pathological findings that were identified throughout the farm and throughout the course of the study

reports of an increase in furunculosis cases in the late periods of spring and early stages of summer when the water temperature is at its highest. The optimal temperature for the growth of *A. salmonicida* is reported to be between 12 and 15°C (22). Akşit and Kum (23) also observed a higher prevalence of *A. salmonicida* isolation between February and April (11-14°C), which is the seasonal transition period, in their studies when the water temperature was 11-18°C. It was observed that the disease progressed in a latent state under these temperatures, with the agents becoming visible as the water temperature increased. In his 1990 article, Rose (24) posited that a water temperature of 15-21°C was

necessary for the occurrence of furunculosis. This finding is corroborated by the observations of Toranzo and Barja (25), who reported the occurrence of furunculosis outbreaks during a period of water temperature between 15 and 16°C. GoldschmitClermant (26) reported the water temperature to be between 8 and 16°C in the study in which they isolated *A. salmonicida* from the liver of naturally infected fish. In contrast, Gültepe and Aydın (27) and Karataş (28) reported the water temperature to be 18°C when they detected the agent in sea water. In addition to the stress placed on the fish by high water temperatures, sudden changes in temperature are significant contributors to the increased incidence of furunculosis (22). The available evidence suggests that the spread and virulence of the disease among enterprises may be attributed to water temperature. Furunculosis disease can manifest in a number of forms, including peracute, acute, subacute-chronic, or latent (intestinal). The peracute form is distinguished by the darkening of the colour of the infected fish and the appearance of vague exophthalmos. In contrast, the acute form is characterised by the presence of haemorrhagic septicaemia and splenic enlargement in fish. In the chronic form, multiple hemorrhagic foci are observed in the muscles and internal organs, as well as enlargement of the spleen and necrosis in the kidney. Large purulent furuncles form at the base of the dorsal and ventral fins of the fish. In cases of latent furunculosis, the sole clinical symptom observed is anus prolapse. The aforementioned findings were identified in the study and are consistent with the presented information (12).

Vitamin D plays a crucial role in a multitude of essential biological functions, encompassing mineral metabolism, cellular proliferation, and cardiovascular physiology (29). Furthermore, research indicates that vitamin D has the potential to induce an antibacterial immune response in mammals (30). The principal natural sources of vitamin D are vitamin D2 and D3, derived from the consumption of fish containing phytoplankton and zooplankton respectively (31). Initially, it was hypothesised that both forms of vitamin D would have a similar effect on human physiology. However, several studies have demonstrated that vitamin D3 is significantly more potent than vitamin D2 (32). In the context of the current study, an analysis of the D3 rate in the commercially available feedstuffs utilised on farms revealed the highest observed rate among those designated as Group A, while the lowest rate was observed among Group C farms. It is also possible that the lower prevalence of the disease on group A farms and the higher prevalence on group C farms may be attributed to the vitamin D3 rate.

An examination of the antimicrobial susceptibility of *A. salmonicida* revealed that florfenicol (30 µg), enrofloxacin (10 µg), amikacin (10 µg) and gentamycin (10 µg) were the most effective agents. The results demonstrated that the tested samples exhibited sensitivity to florfenicol (30 µg) enrofloxacin (10 µg), oxytetracycline (30 µg), amikacin (10 µg) and gentamycin (10 µg). Conversely, neomycin (10 µg),

erythromycin (15 µg) and streptomycin (10 µg) were found to be resistant. Similarly, the *A. salmonicida* strain, derived from a goldfish, exhibited tolerance to ampicillin and vancomycin, while displaying sensitivity to nalidixic acid, chloramphenicol, ciprofloxacin, and tetracycline (33). In a distinct study, *A. salmonicida*, derived from goldfish, exhibited resistance to rifampicin, ampicillin and vancomycin. Conversely, the test demonstrated high sensitivity to ciprofloxacin and nalidixic acid (34). The prevalence of antibiotic resistance among *A. salmonicida* strains is a well-documented phenomenon. This phenomenon can be attributed to the presence of plasmids carrying antibiotic resistance genes (35). While antibiotic resistance in pathogenic bacteria is a significant public health concern, there is a need to ascertain whether the observed resistance is acquired by the introduced genetic material and carried by mobile genetic elements, or if it is intrinsic resistance or mutation-mediated acquired resistance (36). It is therefore evident that the judicious use and selection of antibiotics is of the utmost importance, particularly during an epidemic caused by *A. salmonicida*.

Conclusion

The findings of the study indicate that the prevalence of infection increases in conjunction with an elevation in water temperature. The severity of pathological symptoms correlates with the virulence of the disease. The agent has been detected in fish at temperatures between 15°C and 19°C. Another noteworthy finding is the vitamin D3 rate, which may have been a contributing factor to the disease, potentially in conjunction with temperature. Further research is necessary in order to elucidate the relationship between the D3 rate in feeds and the incidence of furunculosis disease. Furunculosis persists as a significant health concern for cultured fish in Turkey. The disease outbreak on the affected farm was determined to have been caused by the introduction of fish from disparate production units without the implementation of suitable quarantine procedures. It is recommended that fish farmers implement appropriate quarantine procedures prior to introducing new stocks of the same species into their fish ponds. The prophylactic administration of oxytetracycline has been demonstrated to reduce the probability of disease outbreaks. It is imperative to ensure optimal pond hygiene and to eradicate potential intermediate hosts for *A. salmonicida*, such as water lice, within the pond.

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Ocena prisotnosti furunkuloze pri gojenih ribah *Oncorhynchus mykiss*

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Izveleček: Furunkuloza je pomembna in vsesplošna bolezen, ki prizadene sladkovodne in morske ribe po vsem svetu. Bolezen povzroča specifičen etiološki dejavnik, *Aeromonas salmonicida*, ki povzroči nastanek furunkulov ali ulcerativnih mišičnih lezij in drugih kožnih manifestacij, kot so rdeče lise in razbarvanje kože. Obiskanih je bilo 15 ribogojnic postrvi, odvzetih pa je bilo 300 vzorcev 60 osebkov s povprečno živo težo 100-180 gramov. Nato so bile bakterije identificirane s kulturo in MALDI-TOF. Resnost patoloških simptomov je v korelaciji z virulentnostjo bolezn. Povzročitelj je bil odkrit v ribah pri temperaturah med 15°C in 19°C. 40 % rib je kazalo eksoftalmus, 38, 7 % je pokazalo počasno plavanje, 38, 3 % je pokazalo letargično plavanje in 46, 67 % rib je pokazalo izgubo apetita. Prevalenca rib, ki so pokazale bledico škrj, je bila 45 %. Poleg tega je 46, 7 % rib imelo krvavitve v koži in plavutih. Nastanek razjed na koži so opazili pri 36, 7 % rib. Interni pregled je pokazal prisotnost nabiranja krvave tekočine v črevesju rib v 23, 7 %. Poleg tega je 29 % rib pokazalo splenomegalijo, medtem ko je 23, 3 % pokazalo hepatomegalijo in spremembe pigmentacije. Omenjene simptome smo pogosteje opazili na kmetijah skupine A. Preiskava protimikrobne občutljivosti *A. salmonicida* je pokazala, da so občutljivi florfenikol (30 µg), enrofloksacin (10 µg), amikacin (10 µg) in gentamicin (10 µg).

Ključne besede: *Aeromonas salmonicida*; antibiotik; ribogojnice; furunkuloza; razjeda