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Isolation of *Aeromonas bestiarum* in rainbow trout *(Oncorhynchus mykiss)* and determination of antibacterial resistance profile

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Summary

Aeromonas species are microorganisms that can cause clinically significant diseases in fish and humans. We have isolated bacterial pathogens from samples of fish infected with *Aeromonas* species, determined their antibiotic susceptibility profiles and investigated their antibiotic resistance. We examined ten rainbow trout with symptoms of the disease. After necropsy, we isolated and identified bacteria from the lesions in the tissues and organs. We performed culture confirmation and species identification using Matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS). We determined the phenotypic antibiotic susceptibility of *Aeromonas* isolates by the Kirby-Bauer disk diffusion method and evaluated the results according to the standards of the Clinical and Laboratory Standards Institute (CLSI). For phenotypic resistance, we used commercially prepared ready-to-use antibiotic discs of the antibacterial groups tetracyclines, carbapenems, penicillins, amoxicillin/clavulanic acid, folic acid synthesis inhibitors, quinolones, aminoglycosides, amphenicols and macrolides. We also analysed the expression of the antibacterial resistance genes *blaACC, sul1, tetA* and *hfq*. *Aeromonas bestiarum* isolates exhibited phenotypic resistance to ampicillin, penicillin, amoxicillin and erythromycin. Comparative genotypic analysis of the resistance genes *blaACC, sul1* and *tetA* with the

Zusammenfassung

Isolierung von *Aeromonas bestiarum* in einer Regenbogenforelle *(Oncorhynchus mykiss)* und Bestimmung der Antibiotikaresistenz

Aeromonas-Arten sind Mikroorganismen, die sowohl bei Fischen als auch beim Menschen klinisch bedeutsame Erkrankungen verursachen können. Ziel dieser Studie war die Isolierung bakterieller Pathogene aus Proben von mit *Aeromonas*-Arten infizierten Fischen, die Bestimmung der Antibiotikaempfindlichkeitsprofile der *Aeromonas*-Isolate und die Untersuchung ihrer Antibiotikaresistenz. Zehn Regenbogenforellen mit Krankheitssymptomen dienten als Untersuchungsproben. Nach der Autopsie erfolgte die Bakterienisolierung und -identifizierung aus den veränderten Organen. Die kulturelle Bestätigung und die Speziesidentifizierung erfolgten mittels Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS). Die phänotypische Antibiotikaempfindlichkeit der *Aeromonas*-Isolate wurde mittels der Kirby-Bauer-Diskdiffusionsmethode bestimmt, und die Ergebnisse wurden gemäß den Standards des Clinical and Laboratory Standards Institute (CLSI) ausgewertet. Zur Untersuchung der phänotypischen Resistenz wurden kommerziell hergestellte, gebrauchsfertige Antibiotika-Scheiben der folgenden antibakteriellen Gruppen verwendet: Tetracycline, Carbapeneme, Penicilline,

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reference strain revealed the expression of gene segments. We conclude that *Aeromonas bestiarum* agents cause severe infections in rainbow trout. We recommend testing diseased fish for *Aeromonas* and selecting the appropriate antibacterial agent using antibiograms.

Abbreviations: CLSI = Clinical and Laboratory Standards Institute; MALDI-TOF MS= Matrix-assisted laser desorption ionization time of flight mass spectrometry

Amoxicillin/Clavulansäure, Folsäuresynthesehemmer, Chinolone, Aminoglykoside, Amphenicole und Makrolide. Es wurde eine Expressionsanalyse der antibakteriellen Resistenzgene *blaACC, sul1, tetA* und *hfq* durchgeführt. Isolate von *Aeromonas bestiarum* zeigten phänotypische Resistenzen gegen Ampicillin, Penicillin, Amoxicillin und Erythromycin. Eine vergleichende genotypische Analyse der Resistenzgene *blaACC, sul1* und *tetA* mit dem Referenzstamm ergab die Expression von Gensegmenten. Es wurde der Schluss gezogen, dass Erreger von *Aeromonas bestiarum* schwere Infektionen bei Regenbogenforellen verursachen. Daher wird empfohlen, den Erreger aus erkrankten Fischen zu isolieren und das Antibiotikum anhand von Antibiogrammen auszuwählen.

Introduction

The members of the *Aeromonas* (A.) genus are facultative anaerobic microorganisms, Gram-negative, non-encapsulated, sporeless, non-motile or motile with a single polar flagellum and belong to the *Aeromonadaceae* family. They reduce nitrate to nitrite and grow best at temperatures between 22 °C and 28 °C. They can be divided into two main groups based on their motility: motile (*A. hydrophila, A. caviae* and *A. sobria*) and non-motile (*A. salmonicida* and its subspecies *A. bestiarum*) (Martin-Carnahan & Joseph 2005; Chandrarathna et al. 2018).

A. salmonicida, A. hydrophila, A. caviae, A. bestiarum and *A. sobria* cause disease in fish (Martinez-Murcia et al. 2005; Huddleston et al. 2006; Onuk et al. 2017; Zepeda et al. 2017). Motile *Aeromonas* species are present in the aquatic microflora and are pathogenic for various aquatic organisms, as well as for humans. They cause haemorrhagic septicaemia, especially in freshwater fish. Non-motile *Aeromonas* species cause furunculosis in fish.

The taxonomy of *Aeromonas* species is complex and many strains cannot be assigned to subspecies due to their atypical biochemical and genetic properties. For example, a subspecies of *A. salmonicida*, *bestiarum*, was subsequently shown to be an independent species, *A. bestiarum*. However, the 16S rRNA gene sequence of the reference strain of *A. bestiarum* is identical to that of *A. salmonicida* subsp. *achromogenes* and *A. salmonicida* subsp. *masoucida* (Lund et al. 2002; Saavedra et al. 2004; Martínez-Murcia et al. 2005).

The intensive use of antibiotics causes bacteria to develop resistance to them. Misuse or incorrect use accelerates the development of resistance. Vertical and horizontal genetic transfer increases the number of antibiotic-resistant bacteria, reducing the effectiveness of antibiotics. The resulting antibacterial resistance causes the doses of antibiotics used against bacterial diseases in humans and animals to be increased or leads to the introduction of new antibiotics. The use of low-dose antibiotics as growth promoters in animals is banned in most countries because it increases the development of antibacterial resistance but their illegal use may be too widespread to ignore (He et al. 2017; Ayıkol et al. 2020; Muteeb et al. 2023). More precise data can be obtained by the expression analysis of target gene regions (Piotrowska & Popowska 2014; Piotrowska et al. 2017).

We have investigated bacterial pathogens in naturally infected trout with clinical symptoms, isolated *Aeromonas* spp., determined phenotypically the antibacterial susceptibility of the isolates and investigated the antibacterial resistance properties of the bacteria by performing expression analysis of some resistance genes.

Material and Methods

Animal material

Ten samples from trout that died with disease symptoms in January 2024 in trout production facilities in the Karkamış dam lake in southeastern Türkiye were brought to the laboratory of the Microbiology Department of the Faculty of Veterinary Medicine, Harran University under aseptic and cold chain conditions and necropsy procedures were performed. We used the lesioned tissue and organ samples obtained during necropsy (skin, eye, mouth, anus, gill, fin and liver) for bacterial isolation.

Bacterial isolation and identification

We passaged swab and tissue samples taken from the samples onto blood agar base medium (Biolife, Italy) containing 5 % sheep blood. Petri dishes were left for parallel incubation under aerobic conditions at 25 °C and 37 °C. After incubation, we selected colonies from the Petri dishes and purified them by passage, streaking out single colonies. We applied Gram staining (Merck, Germany) to pure bacterial cultures and determined the microscopic morphology. We also performed catalase (Bactident-Merck, Germany), oxidase (Bactident-Merck, Germany), motility and O/F tests (OF test nutrient agar,

Merck, Germany) on the cultures (Quinn et al. 2011). We confirmed the cultures and identified them to species using MALDI-TOF MS (Bruker Daltonics MALDI Biotyper).

Antibiotic susceptibility tests

We determined the phenotypic antibiotic susceptibilities of *Aeromonas* isolates by the Kirby-Bauer Disk Diffusion Method and evaluated the results according to the standards of the Clinical and Laboratory Standards Institute (CLSI) (CLSI M100; CLSI M45). We used commercially prepared ready-to-use antibiotic disks (Bioanalyse, Türkiye) for the groups tetracyclines (doxycycline DO-30, oxytetracycline OTC-30), carbapenems (imipenem IMP-10), penicillins (ampicillin AM-10, penicillin P-10, amoxicillin AX-25), amoxicillin/clavulanic acid AMC-30, folic acid synthesis inhibitors (trimethoprim/sulphamethoxazole SXT-25), quinolones (enrofloxacin ENR-5), aminoglycosides (streptomycin S-10, kanamycin K-30, gentamicin GM-10), amphenicols (chloramphenicol CAP-30) and macrolides (erythromycin E-15, azithromycin AZM-15).

Expression of antibacterial resistance genes

mRNA isolation

We selected a couple of colonies from the *Aeromonas bestiarum* culture isolated from the fin lesion of the fish sample, inoculated them into 10 ml Tryptic Soy Broth (Merck, Germany) and incubated them at 37 °C in an aerobic environment for eight hours. We isolated mRNA from the cultures using the High Pure RNA isolation kit (11828665001/Germany) in accordance with the user manual.

cDNA synthesis from mRNA

We synthesized cDNA from the isolated mRNA using the OneScript plus cDNA synthesis kit (ABM/Canada). We prepared a mix for each sample using all the components included in the kit. 5X RT Buffer 4 µl, DNTP 1 µl, random hexamer primers 1 µl, isolated RNA (2 ng), Onescript Plus Rtase 1 µl and nuclease-free water were added to a volume of 20 µl. cDNA was obtained at 55 ºC for 15 minutes, 85 ºC for 5 minutes, 4 ºC in a Thermalcycler (BIORAD, USA) and measurements taken with a nanodrop spectrometer (Yılmaz et al. 2024).

Real-time PCR

cDNAs were prepared as SybrGreen Master Mix 10µl, forward primer 500 nM 1µl, reverse primer 500 nM 1µl (see Tab. 1), cDNA 5µl, ddH20 3µl, as for the FastStart SybrGreen Master Mix kit (4673484001 Roche, Germany) (Sharma et al. 2018).

The PCR protocol involved 95 °C for 10 min and 45 cycles of amplification at 95 °C for 20 sec, 56 °C for 20 sec and 72 °C for 20 sec. Melting curve analysis was performed at 95 °C for 30 sec, 95 °C for 30 sec, 50 °C for 1 min, 90 °C for continuous reading and 40 °C for 1 min. We used the *Aeromonas bestiarum* ATCC 51108 standard strain for comparison in expression analysis. We selected the hfq gene as it is an accepted reference gene in studies on *Aeromonas* (Soto-Dávila et al. 2022).

Tab. 1: Target genes and primer sequences / Zielgene und Primersequenzen

Gene		Primer	Reference
blaACC	Forward	CACACAGCTGATGGCTTATCTAAAA	Connors et al. 2019; Hayatgheib et al. 2021
	Reverse	CACACAGCTGATGGCTTATCTAAAA	
sul1	Forward	CAGCGCTATGCGCTCAAG	
	Reverse	ATCCCGCTGCGCTGAGT	
tetA	Forward	GCTGTTTGTTCTGCCGGAAA	
	Reverse	GGTTAAGTTCCTTGAACGCAAACT	
hfq	Forward	5′-AAGATTCTGCCCTCAACTGG-3	
	Reverse	5′-GGGCATCGGTCAACAGTAC-3′	

Results

Clinical-macroscopic findings

We observed lethargy, slow movements and loss of appetite, abdominal distension, jaundice and exophthalmos in the eyes, along with various severe deformations in the skin, fins and tails in sick fish. On necropsy, we detected haemorrhagic ulcers in the skin, fins and gills, consistent with the clinical findings. We observed pathological findings in the internal organs, especially in the liver and the kidneys. The liver was enlarged and pale. Diffuse haemorrhagic foci were detected in the kidney and petechial haemorrhages were seen in other organs (see Fig. 1 A, B).

Bacterial isolation and identification

As a result of the cultivations from the muscle, skin and fin lesions, we selected and purified haemolytic colonies that grew intensively in blood agar. Among the bacteria, we classified colonies that were Gram-negative, rod-shaped, facultative anaerobic and oxidase-, catalase- and motility-positive as presumptive *Aeromonas*. We identified the colonies by MALDI-TOF MS analysis as *Aeromonas bestiarum*.

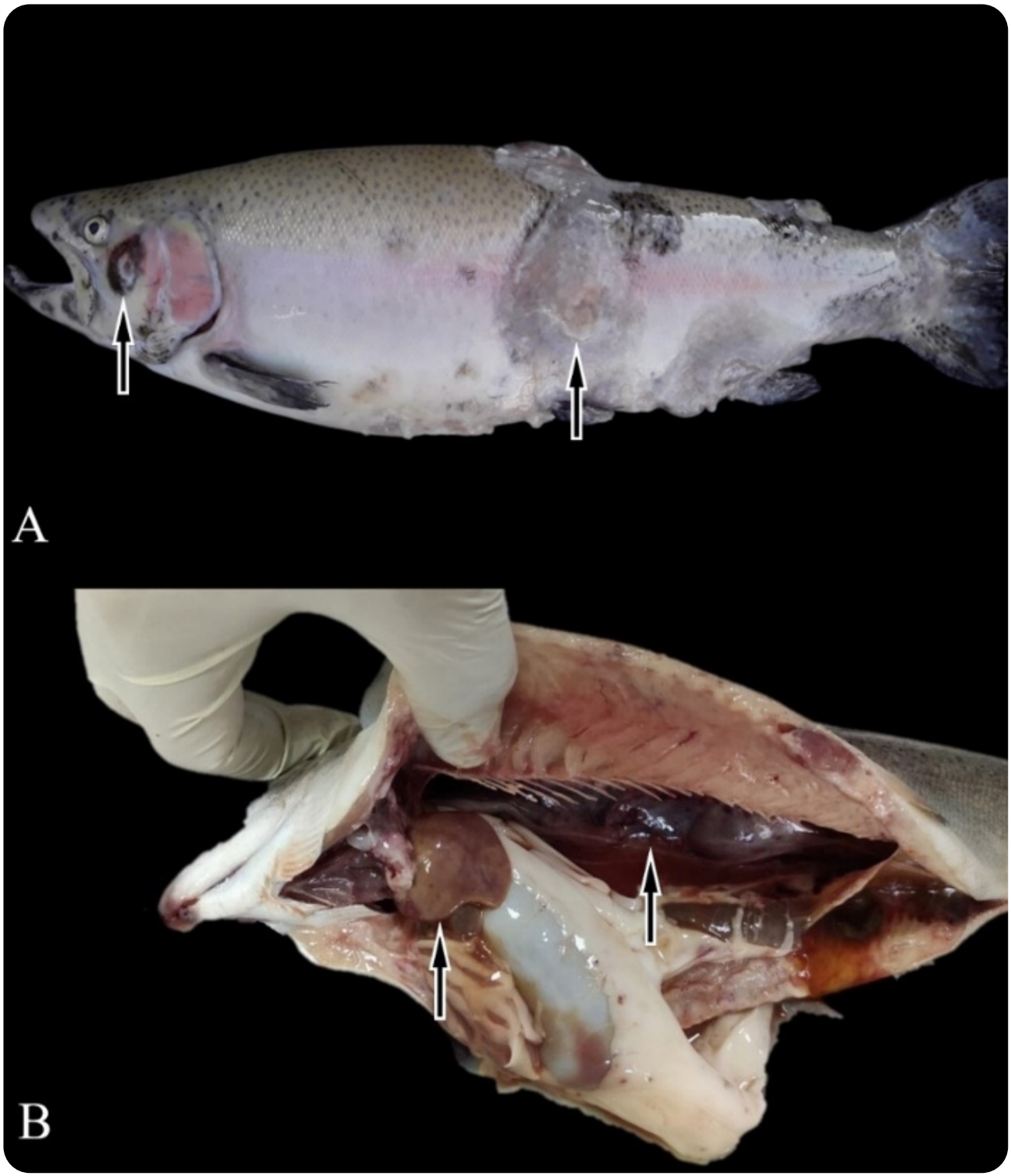

Fig. 1: Macroscopic appearance of rainbow trout: A. Erosive-ulcerative lesions on gills and skin (arrows), B. Necrotic-haemorrhagic changes in liver and kidney (arrows) / Makroskopisches Erscheinungsbild der Regenbogenforelle: A. Erosiv-ulzerative Läsionen an Kiemen und Haut (Pfeile), B. Nekrotisch-hämorrhagische Veränderungen in Leber und Niere (Pfeile)

Antibiotic susceptibility test results

We tested the *Aeromonas bestiarum* isolates for antibiotic susceptibility. All of them were resistant to ampicillin, penicillin, amoxicillin and erythromycin but were sensitive to the other antibiotics investigated (see Tab. 2).

The expression of *blaACC, sul1* and *tetA* genes, which we determined as target genes, was significantly higher in *A. bestiarum* isolated from fish patients than in the control strain (see Fig. 2).

Tab. 2: Antibiogram test results / Ergebnisse des Antibiogrammtests

Group	Antibiotics	Code and Disc Content (µg)	Result
Tetracyclines	Doxycycline	DO-30	S
	Oxytetracycline	T-30	S
ß-Lactames	Imipenem	IMP-10	S
	Ampicillin	AM-10	R
	Penicillin	P-10	R
	Amoxicillin	AX-25	R
	Amoxicillin/ clavulanic acid	AMC-30	S
Folic acid synthesis inhibitors	Trimethoprim/ sulphamethoxazole	SXT-25	S
Quinolones	Enrofloxacin	ENR-5	S
Aminoglycosides	Streptomycin	S-10	S
	Kanamycin	K-30	S
	Gentamicin	GM-10	S
Amphenicols	Chloramphenicol	C-30	S
Macrolides	Erythromycin	E-15	R
	Azithromycin	AZM-15	S
Cephalosporins	Ceftriaxone	CRO-30	S

Discussion

Fish farms suffer from large-scale losses due to infectious diseases and heavy metal poisoning (Dinç & Dörtbudak 2020). *Aeromonas* bacteria can cause various diseases in fish and humans, especially gastroenteritis and septicaemia in immunocompetent and immunodeficient humans (Piotrowska & Popowska 2014). In this study, we isolated bacteria belonging to the *Aeromonas* genus from diseased fish. There have been numerous reports of various *Aeromonas* species isolated from diseased fish in verious countries (Das et al. 2016; Dong et al. 2017; Adah et al. 2024). For example, *Aeromonas hydrophila* and *Pseudomonas fluorescens* have been found in Nile tilapia *(Oreochromis niloticus)* and caused death or clinical findings, with histopathological changes in the liver and kidney. *Aeromonas* species were significantly more abundant than other bacteria in the organs studied and the pathogen had a pronounced effect on the fish (Hal & El-Barbary 2020).

The only *Aeromonas* species we isolated was *Aeromonas bestiarum*. Although *A. bestiarum* is less common than the others species, there have been similar findings in Türkiye and elsewhere. There are numerous reports of the isolation of *Aeromonas* from fish in

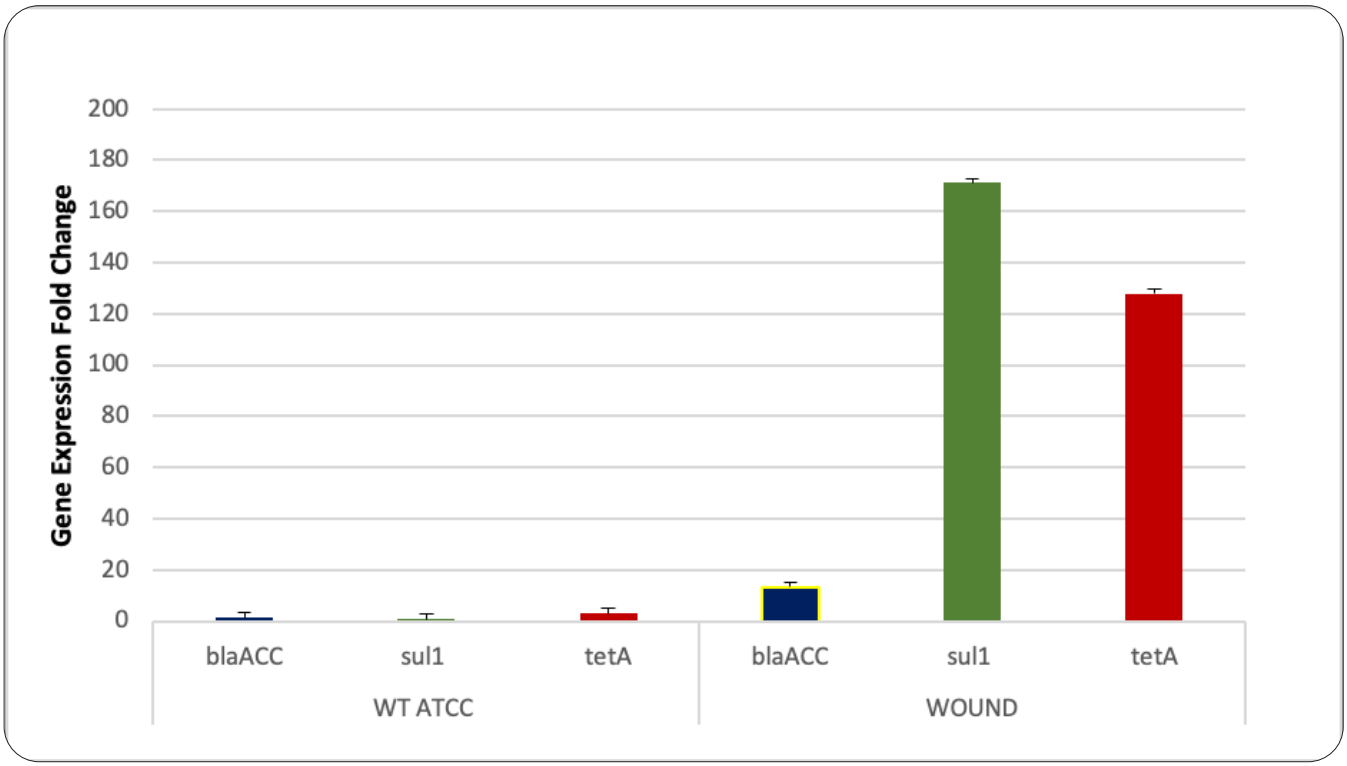

Fig. 2: Expression of antibacterial resistance genes of an *Aeromonas bestiarum* isolate / Analyse der Genexpression der antibakteriellen Resistenz eines *Aeromonas bestiarum*-Isolats

Türkiye (Onuk et al. 2017; Balta 2020; Özdemir & Arslan 2021) but very few of them have identified *A. bestiarum*. Elsewhere, an analysis of 100 fish and water samples in Iran resulted in the isolation of various *Aeromonas* species from the fish samples and a quarter of the isolates were identified as *Aeromonas bestiarum* (Modarres et al. 2014). *A. bestiarum* has subsequently been isolated from humans in Iran, being found in 100 people with burn wounds (Hadi et al. 2019), while the first finding of *A. bestiarum* in farmed carp in Mexico stems from 2010 (Salgado-Miranda et al. 2010). *Aeromonas* has also been isolated from sturgeon and a combination of biochemical tests and molecular methods revealed the agent to be *A. bestiarum* (Utegenova et al. 2024).

The distinction of *Aeromonas* species requires great care due to their phenotypic and genotypic similarities. We did not use biochemical and molecular tests for species distinction as biochemical tests and classical molecular methods have proven inadequate. The taxonomy of the *Aeromonas hydrophila* complex, which includes *A. hydrophila*, *A. bestiarum*, *A. salmonicida* and *A. popoffii* species, is controversial, especially due to the close relationship between *A. salmonicida* and *A. bestiarum*, and none of the biochemical tests considered were able to distinguish these two species from each other. The two may share highly conserved ribosomal operons and 16S rDNA cannot distinguish them due to the high degree of sequence conservation (Martinez-Murcia et al. 2005). There have been studies of the distinctive features of the recA gene: recombinations of this gene give it a high power in distinguishing *Aeromonas* species (Sanglas et al. 2016). However, other groups use other housekeeping gene regions, instead of only this gene region, suggesting that combining multiple gene regions may make the identification of species or subspecies in *Aeromonas* more reliable.

In addition to some biochemical diagnostic methods, we preferred MALDI-TOF MS, which in our hands is a reliable and rapid diagnostic method. It can detect proteins associated with the 16S rRNA gene (Fernández-Bravo & Figueras 2020) and has been used in many laboratories for the bacterial identification of bacteria in recent years, including *Aeromonas* species. When 217 clinical *Aeromonas* isolates previously identified by rpoB sequencing were tested by MALDI-TOF MS, 100 % of them could be confirmed at the genus level and 97 % at the species level (Chen et al. 2014). MALDI-TOF MS correctly identified 98.5% of 65 clinical isolates previously identified by *gyrB* sequencing at the genus level and 92.3% at the species level (Shin et al. 2015). In another study, MALDI-TOF MS correctly identified 98.3% of 179 human isolates to the genus level and 91.1% to the species level, suggesting that MALDI-TOF MS is a useful diagnostic tool as it correctly identifies over 90% of samples (Latif Eugenín 2016). A disadvantage is that the database must be updated to include new species of the *Aeromonas* genus (Fernández-Bravo & Figueras 2020): the number of correct identifications increased after 14 new spectra were added to the MALDI-TOF Biotyper database and the distinction between *Aeromonas bestiarum*, *Aeromonas hydrophila*, *Aeromonas salmonicida, Aeromonas veronii* and *Aeromonas sobria* was correct in over 95% of cases (Pérez-Sánchez et al. 2018).

As the intensive and unconscious use of antibacterial agents accelerates the development of resistance to the substances, antibacterial resistance is becoming an important global health problem. Various phenotypic and genotypic methods have been used to investigate antibacterial resistance in *Aeromonas* bacteria (Leanovich et al. 2025; Moradi et al. 2025; Nagar et al. 2025). The *Aeromonas bestiarum* isolate from sturgeon was resistant to oxacillin, penicillin G, ampicillin, amoxicillin, cefazolin, erythromycin, lincomycin, rifampicin and novobiocin (Utegenova et al. 2024). In another study, *A. bestiarum* was resistant to penicillin, ampicillin, amoxicillin and erythromycin and sensitive only to enrofloxacin and gentamicin. Antibacterial resistance was common in *A. bestiarum* isolates (Tiamiyu et al. 2020).

We found that *A. bestiarum* damages various organs. The isolates are resistant to a range of antibiotics and we report here the expression of some genes that may be related to resistance for the first time. This enabled us to determine the most appropriate antibiotic for the species, which causes damage to commercial fish farms. The pathogen is also important in terms of public health, as the aquatic environment is one of the main paths for the transmission of resistant bacteria from animals to humans and *Aeromonas* causes many diseases in humans, such as bacteraemia, gastroenteritis and even septicaemia in individuals with immune deficiency.

Conclusion for the practice

Experts in the field should consider *Aeromonas* species in the context of fish diseases. Drawing attention to the prevalence of *Aeromonas* on trout, we also emphasize the importance of active isolation in diagnosis to ensure correct treatment. Our findings show the indispensability of the antibiogram test in the effective fight against the disease and the necessity of being aware of antibiotic resistance.

Fazit für die Praxis

Bei der Diagnose von Fischkrankheiten sollte *Aeromonas* als Erreger in Betracht gezogen werden. Um eine erfolgreiche Behandlung bakterieller Infektionen zu ermöglichen, sind die Isolation des Erregers und das Anfertigen eines Antibiogramms nötig.

Conflict of interest: The authors declare no conflict of interest.

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