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Microbial Isolation and Comparison of Antibiotic Resistance Pattern in Rainbow Trout (Oncorhynchus mykiss) from Chaharmahal-va-Bakhtiari Province, Iran (2022-2023)

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ABSTRACT

Background: Increased antibiotic resistance due to the overuse and misuse of antibiotics is a severe threat to both the aquaculture industry and human health. This study aimed to determine the antibiotic resistance pattern of bacteria isolated from rainbow trout.

Materials & Methods: A total of 79 and 120 fish samples were collected in 2022 and 2023, respectively. Swab samples were enriched in TSB (tryptic soy broth) and cultured on TSA (tryptic soy agar). The grown colonies were evaluated for *Aeromonas hydrophila, Lactococcus garvieae, Streptococcus iniae,* and *Yersinia ruckeri.* Antibiotic resistance patterns against 10 common antibiotics were evaluated.

Findings: In this study, four types of bacteria (*A. hydrophila, L. garvieae, S. iniae,* and *Y. ruckeri*) were isolated. The results of antibiotic resistance analysis during 2022-2023 showed that in all isolated bacteria, the percentage of antibiotic resistance against enrofloxacin and florfenicol was higher in 2023 than in 2022.

Conclusion: The isolated bacteria had different resistance patterns, these patterns could be used as a guide for selecting appropriate antibiotics to control infectious diseases in rainbow trout. This finding may be due to improper administration of these antibiotics in rainbow trout production.

Keywords: Drug resistance, *Oncorhynchus mykiss*, Aquaculture

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Introduction

In most countries, marine products are considered as one of the most important sources of protein [1]. Fish meat is very popular due to its low levels of saturated fatty acids and cholesterol, high levels of omega-3 fatty acids, good digestibility, and low price [2]. It has been reported that fish provides about 16% of the world's animal protein [3]. Increasing demand for fish and declining marine reserves have led to a shift to novel aquaculture methods [4]. In modern aquaculture, fish are exposed to many environmental stresses and are at higher risk of disease. Antibiotics are compounds widely used in the aquaculture industry to treat and prevent diseases and sometimes as growth stimulants [5, 6]. Fish production is increasing at a steady rate each year, while antibiotic use is increasing at a much higher rate. Therefore, the widespread therapeutic and non-therapeutic use of antibiotics in fish farming has created very serious problems, including antibiotic resistance in the aquaculture industry, which could reduce antibiotic efficiency [7]. Moreover, considering the structural similarity between antibiotics used in the aquaculture industry and antibiotics used in the medical field, antibiotic resistance in the aquaculture industry could also be a serious threat to human health [8, 9]. Many studies have reported resistance to several antibiotics in bacteria isolated from the fish farm (pool water, sediments, and the fish) [8-10]. It is estimated that about 90% of bacteria isolated from fish farm water pools are resistant to at least one antibiotic, and 20% are resistant to at least five antibiotics [10].

Gufe et al. (2019) reported that bacteria isolated from fish were susceptible to gentamicin (100%)and resistant to lincomycin (100%), ampicillin (81%), penicillin (67%), and erythromycin (65%) [3]. Saharan and colleagues (2020) showed that more than 95% of *Escherichia coli* and *Salmonella* spp. isolated from the feces of Indian fish were resistant to streptomycin. Moreover, *Staphylococcus aureus* showed multiple resistance to antibiotics [11]. In another study conducted by Sicuro et al. (2020) on antibiotic resistance of bacteria isolated from fish kidney, *Aeromonas sobria* was the predominant bacterium, showing resistance to lincomycin, ampicillin, and oxytetracycline [5]. Cunha-Neto et al. (2019) examined the antibiotic resistance of *Salmonella* isolated from fish in Brazil and showed that all isolates were resistant to trimethoprim-sulfamethoxazole combination [12]. In another study by Pena et al. (2019) in Cuba, *Salmonella* and *E. coli* isolated from fish were resistant to ampicillin and tetracycline antibiotics [13]. In recent years, various mechanisms of acquired resistance (such as bacteriophages, transposons, plasmids, and integrons) have been discovered, contributing to the dissemination of resistance genes in bacteria [14]. Shah et al. (2012) reported the presence of resistance genes to amoxicillin and ampicillin antibiotics in cultivated fish in Pakistan and Tanzania [15]. Rainbow trout is a cold-water fish. Increased breeding causes the spread of bacterial diseases and increases the use of antibiotics, subsequently leading to antibiotic resistance

Aquaculture farms always need antibiotics, and the widespread use of antibiotics leads to the prevalence of antibiotic resistance. **Objectives:** Therefore, this study aimed to investigate the antibiotic resistance pattern of bacteria isolated from rainbow trout in Chaharmahal-va-Bakhtiari province, Iran. Most studies have investigated the antibiotic resistance pattern of common bacteria causing fish diseases, and there are little data on more comprehensive antibiotic resistance of bacteria isolated from fish.

Materials and Methods

Sampling and isolation of bacteria: The present study was a cross-sectional descriptive study carried out from January 2022 to December 2023. Sampling was conducted randomly from three rainbow trout aquaculture sites in Chaharmahal-va-Bakhtiari province, Iran. The number of samples taken in 2022 and 2023 were 79 and 120 samples, respectively. To prepare bacterial culture, the back of the gills was cut by sterile scissors, and swab samples were prepared [16].

Swab samples were aseptically blended with tryptic soy broth (TSB) (Merck, Germany) and incubated overnight at 37 °C for enrichment. The cultures were streaked onto tryptic soy agar (TSA) (Merck, Germany) and incubated at 25 °C for 48 hrs. The desired isolates were identified by morphological and biochemical confirmation of their characteristics. Isolation and identification of *Yersinia ruckeri* (*Y. ruckeri*) were done according to the method described by Shaowu and colleagues (2013) [17], *Streptococcus iniae* (*S. iniae*) was isolated according to the study by Russo and colleagues (2006) [18], *Lactococcus garvieae* (*L. garvieae*) was isolated according to the method described by Sharifiyazdi et al. (2010) [19], and *Aeromonas hydrophila* (*A. hydrophila*) was isolated according to the study by Citarasu et al. (2011) [20].

Disk diffusion method: The sensitivity of bacterial strains was evaluated by Kirby-Bauer disk diffusion method according to CLSI (Clinical and Laboratory Standards Institute) guidelines [21]. This method is based on the release of antibiotics into the agar medium and the inhibition of bacterial growth in disk media. Antibiotic discs were purchased from Padtan-Teb Company (Iran). The examined antibiotics included colistin (10 µg), doxycycline (30 µg), enrofloxacin (5 µg), erythromycin (15 µg), florfenicol (30 µg), flumequine (30 µg), fosfomycin (200 µg), linco-spectin (15+200 µg), trimethoprim-sulfamethoxazole (1.25+23.75 µg), and trimethoprim-sulfadiazine. To evaluate antibiotic resistance, bacterial cultures with a0.5 McFarland turbidity (10^8 CFU/mL) were prepared and cultured on Müeller Hinton agar (Merck, Germany) plates, and the plates were incubated at 37 °C for 24 hrs. Then standard antibiotic discs were placed on the culture medium and after 24 hours, the growth inhibition zone diameter was measured. The results were interpreted according to CLSI guidelines (2020) [21] and EUCAST. Version 13.1, 2023 [22].

Statistical analysis: The antibiotic resistant rate in 2022 and 2023 was compared with statistical analysis test (one away ANOVA) in $p<0.05$.

Findings

In this study, 90 and 100 bacterial strains were isolated from 79 and 120 fish samples collected in 2022 and 2023, respectively. The genus and proportion of bacterial strains in 2022 were as follows: *A. hydrophila* 30 (33.33%) strains, *L. garvieae* 10 (11.11%) strains, *S. iniae* 40 (44.44%) strains, and *Y. ruckeri* 10 (11.11%) strains.

Furthermore, out of a total of 100 bacterial strains isolated in 2023, *A. hydrophila* accounted for 20 (20.00%) strains, *L. garvieae* accounted for 10 (10.00%) strains, *S. iniae* accounted for 60 (60.00%) strains, and *Y. ruckeri* accounted for 10 (10.00%) strains. The antibiotic resistance results of *A. hydrophila* obtained in 2022 showed that the highest and lowest resistances were to erythromycin (80%) and enrofloxacin (30%), respectively. The highest resistance of *L. garvieae* was against erythromycin (80%), while its lowest resistance was against colistin (20%). *S. iniae* showed the highest resistance (50%) to sulfadiazine/trimethoprim and sulfamethoxazole/trimethoprim and the lowest resistance to enrofloxacin (20%). *Y. ruckeri* showed the highest and lowest resistance to enrofloxacin (35%) (Tables 1-4).

In 2023, *A. hydrophila*, *L. garvieae*, *S. iniae,* and *Y.ruckeri* showed the highest resistance to lincospectin (100%), enrofloxacin & erythromycin (80%), sulfadiazine/trimethoprim & sulfamethoxazole/trimethoprim (65%), and erythromycin (80%) and the lowest resistance to colistin & doxycycline (0%), lincospectin & flumequine (30%), fosfomycin (35%), and doxycycline (10), respectively (Tables 1-4).

Antibiotic resistance patterns in 2022 and 2023 were very different. The results showed an increase in antibiotic resistance levels against some antibiotics in these two consecutive years. Antibiotic resistance increased in 2023 compared to 2022.

This increase was evident for *A. hydrophila* against flumequine, lincospectin, and sulfadiazine/trimethoprim; *L. garvieae* against colistin, and erythromycin, *S. iniae* against colistin, erythromycin, flumequine, lincospectin, sulfadiazine+trimethoprim, sulfamethoxazole+trimethoprim, and furazolidone; and *Y. ruckeri* against

Table 1) Antibiotic resistance of *A. hydrophila* against different antibiotics in 2022 and 2023 (%)

Antibiotic	2022 (N=30)			2023 (N=20)		
	S	I	R	S	I	R
Colistin	40	0	60	80	20	0
Doxycycline	30	10	60	30	70	0
Enrofloxacin	40	30	30	10	50	40
Erythromycin	10	10	80	0	30	70
Florfenicol	40	10	50	30	10	60
Flumequine	30	30	40	30	0	70
Fosfomycin	30	30	40	30	50	20
Linco-spectin	20	30	50	0	0	100
Trimethoprim-sulfadiazine	20	30	50	20	30	50
Trimethoprim-sulfamethoxazole	30	30	40	40	0	60

* S: sensitive, I: intermediate (semi-resistant), R: resistant

Table 2) Antibiotic resistance of *L. garvieae* against different antibiotics in 2022 and 2023 (%)

Antibiotic	2022 (N=10)			2023 (N=10)		
	S	I	R	S	I	R
Colistin	70	10	20	30	10	60
Doxycycline	20	30	50	20	30	50
Enrofloxacin	40	20	40	10	10	80
Erythromycin	20	0	80	10	10	80
Florfenicol	40	20	40	20	10	70
Flumequine	10	40	50	60	10	30
Fosfomycin	10	20	70	20	30	50
Linco-spectin	10	40	50	40	30	30
Trimethoprim-sulfadiazine	10	20	70	20	30	50
Trimethoprim-sulfamethoxazole	20	10	70	20	20	60

* S: sensitive, I: intermediate (semi-resistant), R: resistant

Table 3) Antibiotic resistance of *S. iniae* against different antibiotics in 2022 and 2023 (%)

Antibiotic	2022 (N=40)			2023 (N=60)		
	S	I	R	S	I	R
Colistin	75	0	25	30	10	60
Doxycycline	55	5	40	30	30	40
Enrofloxacin	60	20	20	40	15	45
Erythromycin	25	30	45	10	30	60
Florfenicol	45	20	35	50	5	45
Flumequine	50	20	30	35	15	50
Fosfomycin	30	25	45	35	30	35
Linco-spectin	35	25	40	30	25	45
Trimethoprim-sulfadiazine	40	10	50	20	15	65
Trimethoprim-sulfamethoxazole	40	10	50	20	15	65

* S: sensitive, I: intermediate (semi-resistant), R: resistant

Table 4) Antibiotic resistance of *Y. ruckeri* against different antibiotics in 2022 and 2023 (%)

Antibiotic	2022 (N=10)			2023 (N=10)		
	S	I	R	S	I	R
Colistin	0	50	50	50	10	40
Doxycycline	40	20	40	30	60	10
Enrofloxacin	45	20	35	20	30	50
Erythromycin	25	25	50	10	10	80
Florfenicol	10	25	65	20	5	75
Flumequine	30	10	60	30	10	60
Fosfomycin	20	40	40	40	10	50
Linco-spectin	10	30	60	10	20	70
Trimethoprim-sulfadiazine	10	30	60	10	20	70
Trimethoprim-sulfamethoxazole	10	20	70	20	10	70

* S: sensitive, I: intermediate (semi-resistant), R: resistant

Table 5) Percentage of antibiotic resistance of isolated bacteria in 2022 and 2023 (mean±SD)

Bacteria/Year	2022	2023	*P* Value
A. hydrophila	50.00±14.14	47.00±32.33	.79
L. graviae	54.61±17.13	53.84±16.09	.90
S. iniae	37.69±9.26	50.76±9.96	.002
Y. ruckeri	56.93±13.31	55.38±19.08	.81

erythromycin, fosfomycin, lincospectin, and sulfadiazine+trimethoprim. Increased resistance to enrofloxacin and florfenicol was observed in all isolated bacteria in these two consecutive years.

A comparison of antibiotic resistance between 2022 and 2023 showed that antibiotic resistance in 2023 was higher in *S. Iniae* and for other bacterial spp against some antibiotics e.g. enrofloxacin and florfenicol, compared to 2022 (Table 5). Antibiotic resistance in *S. iniae* was significantly higher in 2023 than in 2022 (p= .002), while no significant difference was observed for other bacteria (Tables 1-4).

Discussion

In this study, four prevalent aquaculture pathogens were isolated from rainbow trout in Chaharmahal-va-Bakhtiari province, western Iran. The prevalence and importance of these pathogens have been reported by some researchers in Iran. Soltani et al. (2013) identified *S. iniae* and *L. garvieae* in rainbow trout breeding sites of Kohgiluyeh-va-Boyer-Ahmad and Chaharmahal-va-Bakhtiari provinces, respectively [22]. Sharifiyazdi et al. (2010) also identified *S. iniae* and *L. garvieae* in rainbow trout [19], which is consistent with the present study results. Similarly, Karsidani et al. (2010) isolated *S. iniae* from 59.2% of rainbow trout samples in seven provinces of Iran [23].

Antibiotic resistance has been increasingly reported in various animal species. For example, Mohammadi and Karimi Dehkordi (2019) documented notable antibiotic resistance patterns in *Salmonella* serotypes isolated from broiler chickens. In their study, the prevalence of *Salmonella* in broilers was reported to be 33%, and *S. enteritidis* was identified as one of the most important antibiotic-resistant serotypes [24].

Antibiotics are widely used in the aquaculture industry because of their high efficiency in the treatment and prevention of infectious diseases. However, their adverse effects could also cause damages to aquaculture and even humans. Increased antibiotic resistance in bacteria is one of the adverse effects of the increased use of antibiotics, which reduces antibiotic efficiency and could lead to the transmission of resistance to humans and the environment [8, 9]. Many studies have been carried out on antibiotic resistance patterns of bacteria isolated from trout. The present study results could be used by health policymakers in the aquaculture industry.

One of the fundamental principles regarding antibiotic resistance is to monitor antibiotic resistance patterns in various regions and time zones. The difference in resistance patterns is probably due to differences in the type, amount, and duration of antibiotic use [6]. In this line, in the present study, resistance varied depending on the type of antibiotic, bacteria, and year. In this study, the antibiotic resistance frequency was compared in 2022 and 2023. The rate of antibiotic resistance can be confounded by different sample size in these years (79 vs 120).

Some studies have been carried out on antibiotic resistance in rainbow trout in Iran. In a study conducted by Faeed and Ramezani (2018) in Guilan province, the antibiotic resistance percentage of *S. iniae* to doxycycline, florfenicol, and erythromycin was reported to be 25, 54, 0, and 20%, respectively [25]. In another study by Soltani et al. (2025) on the antibiotic resistance pattern of *S. iniae* in rainbow trout in Kurdistan province of Iran, the highest resistance was related to lincomycin, bacitracin, flumequine, and sulfadiazine [26]. In the present study, the highest antibiotic resistance of *S. iniae* in 2022 and 2023 was against sulfonamide compounds. This finding contrasts with the results of aforementioned studies [25, 26]. This difference may be the result of various antibiotic therapy schedules used in

different provinces.
Shahrani and colleagues (2014) highlighted that the highest resistance of *L. garvieae* isolated from rainbow trout in Chaharmahal-va-Bakhtiari province was related to enrofloxacin (65.4%) [27].
In a study by Yari and colleagues (2017), all *Lactococcus* and *Streptococcus* isolates from rainbow trout aquaculture farms in Ilam province (Iran) were resistant to erythromycin (23%), streptomycin (35%), and oxytetracycline (31%) [28]. In another study by Raissy and Moumeni (2016), L. garvieae isolates from rainbow trout in Chaharmahal-va-Bakhtiari province of Iran were resistant to tetracycline (n=19, 79.1%) and erythromycin (n=9, 37.5%)." [29]. The present study results are in agreement with those of previous studies regarding erythromycin resistance of *L. garvieae* in trout.
Enrofloxacin was an effective antibiotic against most of the isolated bacterial species, and the highest sensitivity was related to this antibiotic, which is similar to the finding of Soltani et al. (2023) in Iran [30]. Most of the isolated bacterial species showed the highest resistance to erythromycin, which may be due to the widespread use of this antibiotic due to its lower price and greater availability in trout farms. However, erythromycin is an effective drug against streptococcosis and lactococcosis [29], which have a high prevalence in trout farms. Therefore, frequent and more use of this drug has led to the emergence of high resistance.
Ture and Boran (2015) reported erythromycin resistance of *L. garvieae* in Turkish rainbow trout [31]. Duman et al. (2020) examined antibiotic resistance of *L. garvieae* in Turkish rainbow trout and reported 2.8, 17.8, and 10.7% resistance to erythromycin, florfenicol, and tetracycline, respectively [32]. The present study findings regarding antibiotic resistance of *L. garvieae* are in accordance with the findings of these studies representing higher resistance to erythromycin.
The present study results along with reports in the literature confirm that resistance to common antibiotics used in aquaculture is increasing. It should be noted that bacterial resistance patterns in various regions may differ.

Conclusion

In conclusion, antibiotic resistance significantly increased in 2023 compared to 2022, only in *S. iniae*. Therefore, it is necessary to check and evaluate resistance of bacterial agents to common antibiotics every year. This knowledge will guide veterinarians in choosing appropriate initial antibiotics to treat infections.

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Authors' contributions: Meryam Karimi-Dehkordi designed and supervised the study, analyzed the data, and wrote the

manuscript. Mohammad Ghasemi-Shamsabadi performed the experimental procedures and contributed to data collection and analysis. Majid Gholami-Ahangaran provided scientific guidance, reviewed the manuscript, and approved the final version for submission.

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