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Emergence of Multidrug-Resistant Bacterial Pathogens in Cultured Rainbow Trout (*Oncorhynchus mykiss*): Phenotypic and Molecular Insights

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Abstract

The emergence of antimicrobial resistance in aquaculture poses a significant threat to both fish health and public safety. In this study, rainbow trout (*Oncorhynchus mykiss*) from the Kokernag trout-rearing unit were collected to isolate and characterize bacterial pathogens, assess antimicrobial susceptibility, and detect antibiotic resistance genes in the fish. Based on phenotypic and genotypic characterization, six bacterial isolates, including *Aeromonas sobria, Escherichia coli, Flavobacterium psychrophilum, Pseudomonas spp., Staphylococcus spp.,* and *Yersinia ruckeri,* were identified. Antimicrobial susceptibility testing revealed variable resistance patterns, with high resistance observed against ampicillin, oxytetracycline, and erythromycin, whereas florfenicol, gentamicin, ceftazidime, and meropenem remained effective. Molecular screening confirmed the presence of sul1 and blaTEM antibiotic resistance genes among the isolates. These findings highlight the prevalence of multidrug-resistant bacteria in trout aquaculture, underscoring the importance of prudent antibiotic use, continuous surveillance, and integrated disease management strategies to safeguard both aquaculture productivity and public health.

Keywords: Aquaculture, Fish Pathogens, Antimicrobial Resistance, One Health, Antibiotic Resistance Genes.

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Introduction

Beginning with the cultivation of rainbow trout (*Oncorhynchus mykiss*) around 1900, aquaculture has become a significant industry in Kashmir. *Oncorhynchus mykiss* is highly valued in aquaculture due to its rapid growth, rich nutritional profile, and ease of culture. As the most widely farmed trout species globally, it holds substantial commercial significance. In Northern India, the species is extensively reared in cold-water habitats to cater to the increasing demand for quality fish protein. The expansion of trout farming in Jammu and Kashmir is evident from the establishment of 59 dedicated trout-rearing facilities (Singh et al., 2017). However, this rapid development is accompanied by persistent vulnerability of cultured trout to infectious agents, including viruses, bacteria, and fungi. Among these, bacterial diseases are particularly detrimental and can severely compromise production, especially when fish experience stress resulting from overcrowding, deteriorating water quality, inadequate nutrition, or poor management practices. Bacterial infections pose a significant challenge to the aquaculture sector, often leading to substantial mortality losses (Deekshit et al., 2023). The widespread use of antibiotics in aquaculture, mainly for managing infectious diseases, has significantly contributed to the escalating problem of antimicrobial resistance (AMR). Although antibiotics play a crucial role in controlling bacterial outbreaks, their frequent and often excessive use in fish farming leads to the release of unmetabolized residues into surrounding water bodies. This environmental exposure promotes the selection and proliferation of antibiotic-resistant bacteria, posing risks not only to aquatic organisms but also to human health (Reverter et al., 2020). Resistance identified in aquaculture-associated bacteria has been shown to transfer to human pathogens, thereby intensifying the challenge of treating bacterial infections. The dissemination of resistance genes, particularly through interconnected aquatic systems, is of growing concern as it facilitates the emergence of hard-to-treat resistant strains (Karunasagar et al., 2012; Zhao et al., 2021). The purpose of this study is to present a thorough investigation of the AMR profiles of the main bacterial pathogens that influence the aquaculture of trout fish in Kashmir, North India. Additionally, molecular identification and the detection of specific antibiotic resistance genes (ARGs) will be carried out to elucidate the genetic basis underlying the observed resistance patterns. Together, these approaches will provide a clearer understanding of the emerging antimicrobial resistance landscape within the region's aquaculture systems.

Materials and Methods

Sample Collection and Bacterial Isolation

Oncorhynchus mykiss samples were collected from the Kokernag trout-rearing unit, a key aquaculture facility in Jammu and Kashmir, using standard aseptic procedures. Following collection, each specimen was examined, and any visible signs or symptoms indicative of infection were carefully documented. The samples were then properly labeled and immediately placed in sterile containers for transport. They were transferred to the laboratory without delay to ensure sample integrity and minimize the risk of contamination. Swabs were first obtained from the external integument and skin lesions of the fish. The specimens were then aseptically dissected to obtain additional swabs from internal organs of interest, including the liver, gut, and kidney. All swabs were immediately inoculated onto Tryptic Soy Agar (TSA) plates and incubated at 37 °C for 18–24 hours. Further purification of bacterial growth was achieved using the streak plate technique to obtain isolated colonies. The preliminary identification of pure bacterial isolates was done on the basis of colony morphology and Gram staining.

Biochemical Characterization

Purified bacterial isolates were further subjected to a panel of biochemical assays, including indole production, nitrate reduction, motility, catalase activity, oxidase activity, urease production, and carbohydrate fermentation. All tests were conducted according to the manufacturer's recommended guidelines and standard microbiological protocols.

Antimicrobial Susceptibility Testing (AST)

AST of the bacterial isolates was performed on Mueller–Hinton Agar (MHA) using the Kirby–Bauer disk diffusion method in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2024). Freshly cultured bacterial cells were suspended in sterile normal saline and adjusted to the 0.5 McFarland standard. The standardized inoculum was evenly spread over the surface of MHA plates using a sterile cotton swab. Commercial antibiotic discs representing commonly used aquaculture and clinical antibiotics were applied, including ampicillin (AMP), oxytetracycline (OTC), ceftazidime (CTZ), enrofloxacin (ENR), erythromycin (ERY), oxolinic acid (OXA), florfenicol (FLO), gentamicin (GEN), and meropenem (MER). The plates were incubated at 37 °C for 18–24 hours. Following incubation, the diameters of the inhibition zones were measured in millimeters using a vernier caliper. Based on CLSI breakpoint criteria, isolates were categorized as susceptible (S), intermediate (I), or resistant (R).

Molecular Identification of Bacterial Isolates

For genomic DNA extraction, freshly cultured bacterial colonies grown in nutrient broth were processed using the commercial DNA Extraction Kit (Himedia), following the manufacturer's protocol. Amplification of the extracted DNA was performed using conventional PCR. Molecular identification was performed by targeting the 16S rRNA gene using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1429R (5'-GGTTACCTTGTTACGACTT-3'). A 20 µL PCR reaction mixture was prepared containing 1 µL of DNA template, 0.4 µL each of dNTPs, forward primer, and reverse primer, 2 µL of 10X buffer, 0.2 µ L of Taq DNA polymerase, and the remainder of Milli-Q water. The 16S rRNA gene was amplified using an initial denaturation at 95 °C for 5 minutes, followed by denaturation at 94 °C for 30 seconds. The annealing step was carried out at 55 °C for 1 minute. PCR products were sequenced to confirm species-level identity. Obtained sequences were compared with NCBI GenBank entries using BLAST for taxonomic confirmation.

Isolation of Antimicrobial Resistance Genes

Genes associated with antimicrobial resistance were amplified using gene-specific primers (**Table 1**) through PCR. The target genes selected for amplification included blaTEM, blaOXA48, sul1, gyrA, tetA, and aadA1, using genomic DNA from the isolated fish pathogens as the template. Multiple PCR reactions were prepared, and each target gene was amplified using its respective primer set. The initial denaturation temperature for blaTEM, blaOXA48, Sul1 gyrA, and tetA genes was 95° C for 5 min. For the aadA1 gene, the initial denaturation was performed at 95 °C for 3 minutes. Denaturation for blaTEM, blaOXA48, and aadA1genes was carried out at 95 °C for 30 s, for tetA and sul1 at 94 °C for 60 s, and gyrA gene at 95 °C for 50 s. The annealing temperature for each gene amplification is given in Table 1. PCR products were electrophoresed on agarose gels, and the presence of amplicons of the expected size was recorded as positive for the respective resistance gene. A 100-bp DNA ladder was used to determine the sizes of the PCR products.

Table 1. Primer sequences used for PCR amplification of antimicrobial resistance genes

Primer Pair	Direction	Primer Sequence (5′ → 3′)	Annealing temperature	Amplicon Size (bp)
blaTEM	Forward (F)	GAGACAATAACCCTGGTAAAT	60 °C	459
	Reverse (R)	AGAAGTAAGTTGGCAGCAGTG		
blaOXA48	Forward (F)	GCGTGGTTAAGGATGAACAC	60 °C	438
	Reverse (R)	CATCAAGTTCAACCCAACCG		
sul1	Forward (F)	TTCGGCATTCTGAATCTCAC	50 °C	822
	Reverse (R)	ATGATCTAACCCTCGGTCTC		
gyrA	Forward (F)	TACCGTCATAGTTATCCACGA	61.5 °C	313
	Reverse (R)	GTACTTTACGCCATGAACGT		
tet A	Forward (F)	GGTTCACTCGAACGACGTCA	56°C	577
	Reverse (R)	CTGTCCGACAAGTTGCATGA		
aadA1	Forward (F)	TATCCAGCTAAGCGCGAACT	58 °C	447
	Reverse (R)	ATTTGCCGACTACCTTGGTC		

Results

Isolation of bacterial strains

Following inoculation of external lesion swabs and internal organ samples (liver, gut, and kidney) onto TSA and incubation at 37 °C for 18–24 hours, A total of six bacterial pathogens were isolated from the rainbow trout. These bacterial strains, which exhibited visible growth, were subsequently used for further analysis.

Gram staining and biochemical characteristics

Gram staining confirmed that five isolated bacterial strains-S1, S2, S4, S5, and S6-were Gram-negative, whereas the S3 bacterial strain was Gram-positive. The results of the biochemical analyses are summarized in **Table 2**. The biochemical profiles observed for each species are consistent with previously documented reports.

Table 2. Biochemical tests performed on the isolated bacterial pathogens.

Pathogen	Catalase	Oxidase	Motility	Indole	Nitrate Reduction	Urease	Carbohydrate Utilization
A. sobria	+	–	+	–	+	–	+
E. coli	+	–	+	+	+	–	+
F. psychrophilum	Weak/–	+	– /Weak	–	–	–	+
Pseudomonas spp.	+	+	+	–	Variable	–	+
Staphylococcus sp.	+	–	–	–	Variable	+	+
Y. ruckeri	+	–	+	–	+	–	+

Molecular identification via 16S rRNA sequencing

For species-level identification, representative isolates from each morphological group were randomly selected for molecular characterization through 16S rRNA gene sequencing (**Figure 1**). High-quality PCR amplicons were obtained and subsequently sequenced to determine their genetic identity. The resulting sequences were analyzed using the BLAST tool available in the NCBI database. Comparative sequence analysis revealed ≥98% nucleotide similarity with established reference strains, confirming the taxonomic identity of the isolates. Specifically, the sequences showed closest alignment with *Aeromonas sobria*, *Escherichia coli*, *Flavobacterium psychrophilum*, *Pseudomonas* spp., *Staphylococcus* sp., and *Yersinia ruckeri*. These molecular findings were fully consistent with the morphological, Gram staining, and biochemical profiles obtained for the corresponding isolates, thereby validating the reliability of the identification approach used in this study.

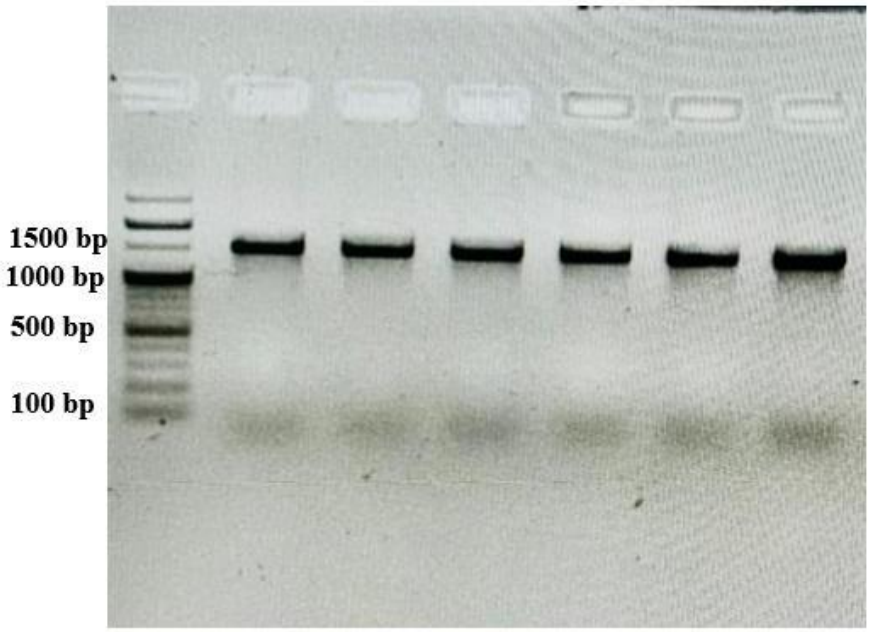

Figure 1: Electrophoresis of 16S rRNA genes (Marker 100 bp, 16S rRNA: 1500 bp).

Antimicrobial susceptibility patterns

Antimicrobial susceptibility testing revealed that the tested antibiotics exhibited distinct and species-specific susceptibility patterns against the isolated pathogens. *Aeromonas sobria* showed resistance to ampicillin, oxytetracycline and erythromycin, whereas gentamicin, florfenicol, ceftazidime, meropenem, and enrofloxacin were predominantly effective. *Escherichia coli* showed resistance to ampicillin, ceftazidime, and oxytetracycline, while meropenem, gentamicin, and enrofloxacin were significantly effective. *Flavobacterium psychrophilum* demonstrated high susceptibility to florfenicol and meropenem, whereas reduced sensitivity towards oxytetracycline, oxolinic acid, and enrofloxacin. Meropenem, gentamicin, and ceftazidime exhibited the highest activity against *Pseudomonas* spp., whereas ampicillin, oxolinic acid, enrofloxacin, and oxytetracycline showed resistance. *Staphylococcus* was largely susceptible to florfenicol, gentamicin, ceftazidime, and meropenem, although resistance to ampicillin and erythromycin was observed in some strains. *Yersinia ruckeri* isolates were mostly susceptible to oxytetracycline, florfenicol, enrofloxacin, ceftazidime and meropenem, whereas moderate resistance was found against oxolinic acid and oxytetracycline.

Detection of antibiotic resistance genes

Molecular screening of antibiotic resistance genes using PCR followed by agarose gel electrophoresis confirmed the presence of sul1 and *bla*TEM among the isolated pathogens **(Figure 2).** It is noteworthy that no other antibiotic resistance genes, including blaOXA-48, gyrA, tetA, and aadA1, were detected among the isolates analyzed.

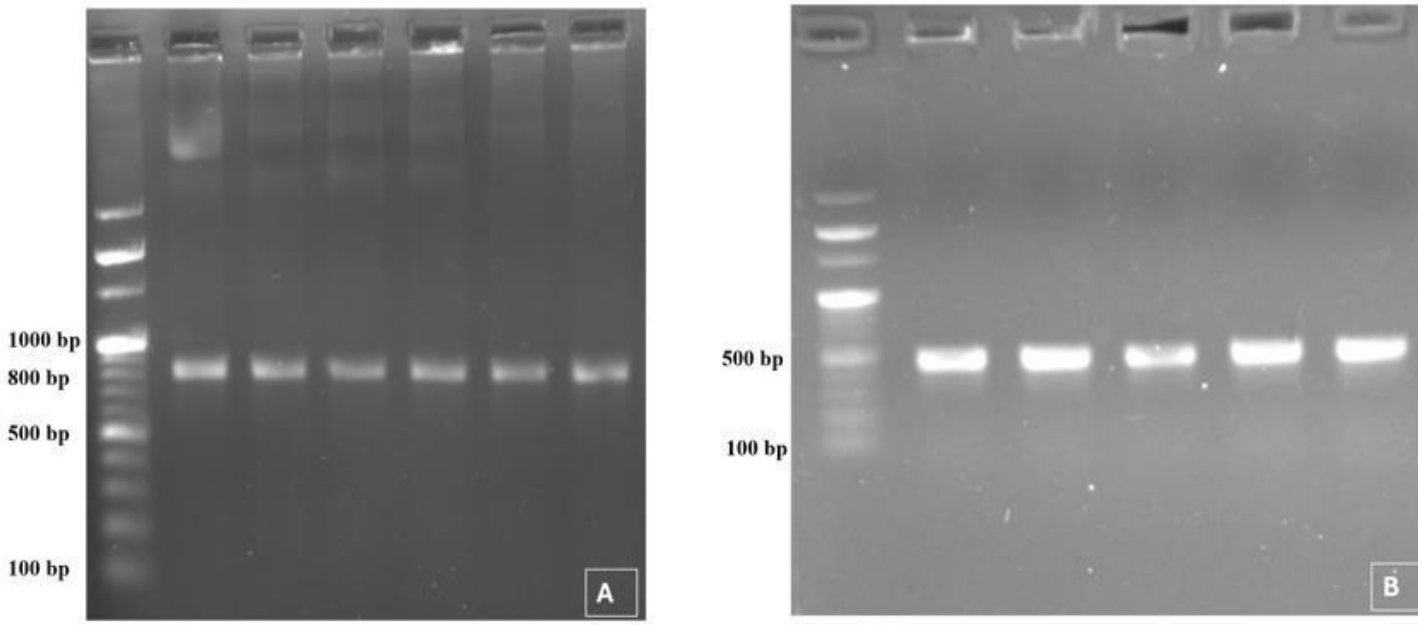

Figure 2: Electrophoresis of antibiotic-resistant genes :(A) sul1 and (B) blaTEM (marker 100 bp, sul1:822 bp and blaTEM: 459 bp).

Discussion

The emergence of AMR in fish pathogens presents a growing challenge for both aquaculture sustainability and public health. Oncorhynchus *mykiss*, one of the most widely farmed cold-water species, is particularly vulnerable to a range of bacterial infections that can significantly impact survival, productivity, and economic viability (Boyraz et al., 2024). In recent years, increasing reports of multidrug-resistant (MDR) bacteria isolated from aquaculture environments have raised serious concerns regarding the overuse and misuse of antibiotics in fish farming (Hossain et al., 2022; Thornber et al., 2022). Bacteria are primary agents of disease in living organisms, and the rising incidence of antibiotic resistance among them poses a serious global threat. Aquatic environments serve as an important route for the transfer of antibiotic resistance genes from environmental microbes to human pathogens, thereby contributing to the emergence of resistant strains and reducing the effectiveness of available therapies. Evidence from epidemiological and molecular studies indicates that fish-associated pathogens can transmit resistance determinants to human-related bacteria. Because aquatic organisms are constantly in contact with water, they are naturally prone to bacterial exposure (Ahmad et al., 2024; Qurratulan et al., 2024).

The present study provides a comprehensive assessment of bacterial pathogens associated with diseased Oncorhynchus mykiss from the Kokernag trout-rearing unit, combining classical microbiological methods with molecular identification and antimicrobial resistance profiling. Six pathogenic bacterial species, such as *Aeromonas sobria, Escherichia coli, Flavobacterium psychrophilum, Pseudomonas* spp., *Staphylococcus* spp., and *Yersinia ruckeri* were isolated from both external lesions and internal organs (Miller & Harbottle, 2018; Mir et al., 2022). The coexistence of multiple pathogenic taxa highlights the complex microbial ecology of aquaculture systems and underscores the potential for polymicrobial infections in farmed fish, which is consistent with earlier findings and studies (Austin & Austin, 2016)The detection of *A. sobria, F. psychrophilum,* and *Y. ruckeri*, all well-known etiological agents of hemorrhagic septicemia, bacterial coldwater disease, and enteric redmouth disease, respectively, reflects the susceptibility of trout to opportunistic environmental bacteria, particularly under intensive aquaculture conditions.

Antimicrobial susceptibility testing revealed marked variability in resistance profiles among the isolates, indicating species-specific responses to drugs. The high resistance of *A. sobria* and *E. coli* to ampicillin, oxytetracycline, and erythromycin aligns with global trends reporting increasing resistance to commonly used antibiotics in aquaculture (Landor et al., 2024). The observed susceptibility of most isolates to florfenicol, gentamicin, ceftazidime, and meropenem suggests that these antibiotics retain therapeutic potential; however, reliance on such drugs, particularly antibiotics like meropenem, raises concerns about selection pressure and the possible spread of resistance determinants to aquatic and human-associated microbiota (Ben et al., 2019).

The molecular detection of sul1 and blaTEM resistance genes further supports these phenotypic resistance patterns. The sul1 gene, commonly associated with class 1 integrons, confers resistance to sulfonamides and is frequently reported in aquatic environmental

bacteria due to historical and ongoing exposure to antibiotics. Similarly, blaTEM is one of the most widespread β-lactamase genes and has previously been documented in aquaculture-associated Enterobacteriaceae, reflecting potential anthropogenic contamination or horizontal gene transfer events. The absence of other tested genes (e.g., tetA, aadA1, gyrA, and blaOXA-48) suggests a lower prevalence of multi-resistance determinants within the study isolates; nonetheless, continuous surveillance is warranted given the dynamic nature of aquatic resistomes (Helsens et al., 2020; Miller & Harbottle, 2018). Collectively, these findings highlight significant public health and aquaculture management implications. The presence of multidrug-resistant bacteria in trout farms emphasizes the urgent need for prudent antibiotic use, implementation of biosecurity measures, and integration of molecular surveillance tools to monitor emerging resistance trends. Sustainable disease control strategies must prioritize vaccination, improved husbandry practices, and regular screening to mitigate pathogen spread and preserve antibiotic efficacy.

Conclusion

This study highlights the emergence of multidrug-resistant bacterial pathogens in cultured *Oncorhynchus mykiss* from Kashmir aquaculture. Phenotypic resistance to commonly used antibiotics, alongside the detection of transferable resistance genes (sul1 and blaTEM), underscores the role of aquaculture as a reservoir of AMR. Given the risk of antibiotic-resistant gene transmission to humans and the environment, urgent preventive measures such as prudent antibiotic use, routine pathogen monitoring, enhanced farm biosecurity, and vaccination are essential to mitigate disease outbreaks and limit the spread of AMR under a One Health framework.

Author Contributions (CRediT Statement)

Aamir Majeed: Conceptualization, Investigation, Data curation, Formal analysis, Writing – original draft. **Ishfaq Ahmed:** Writing – review & editing. **Shigufta Ali:** Data curation, Writing – review & editing. **Tanveer Ahmad Mir:** Supervision, Laboratory analysis, Methodology, Writing – review & editing. **Muzamil Liakat Mir:** Visualization, Writing – review & editing.

Conflict of Interest

Authors declare no conflict of interest.

Data availability

Data sets used/analysed during the current study will be available from corresponding authors on request.

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