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The Fleming Fund

Pilot AMR Surveillance in Healthy Aquatic Animals in Pakistan (Inland Farmed Fish)

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HEALTH SECURITY PARTNERS

Shifa International Hospital Faisalabad

DISCLAIMER STATEMENT ON THE PRELIMINARY NATURE AND USE OF THIS REPORT

This document presents findings from the **"Pilot Active AMR Surveillance in Healthy Inland Farmed Fish"** conducted from November 2024 to July 2025.

It is crucial to note that:

1. This was a **pilot study** designed to establish methods, build capacity, and generate initial baseline data.
2. The findings are based on a **limited sample size** (n=364 fish from 9 sentinel sites) and are **not representative** of all aquaculture systems or species in Pakistan.
3. The antimicrobial susceptibility testing was performed using phenotypic methods only. **Absence of molecular confirmation** (e.g., PCR for resistance genes) means the genetic mechanisms of resistance are not characterized in this report.

Therefore, the data and conclusions in this report are strictly preliminary. They are intended for:

- Informing the design of future, more robust surveillance studies.
- Guiding stakeholder discussions on AMR in the One Health context.
- Strengthening national technical capacity.

This report and its data must not be used for:

- Making export-related decisions or certifications.
- Enacting immediate regulatory measures against the aquaculture sector.
- Drawing definitive national-level estimates of AMR prevalence.

Foreword

Food security is a key factor in defeating malnutrition and improving a country's socioeconomic status. To overcome nutrition deficiencies, fisheries contribute a significant amount of animal protein to the diets of people worldwide. This source of protein is a highly nutritious meat source high in omega-3 compared with livestock and poultry. Aquaculture also plays a vital role in national economic development and the global food supply. However, this sector may contribute to the spread of antimicrobial resistance (AMR), one of the most pressing global One Health challenges, due to excessive therapeutic and prophylactic antimicrobial use (AMU).

To curtail AMR, a country needs a strong monitoring and surveillance system for AMR across both veterinary and human sectors. The Ministry of National Food Security and Research (MoNFS&R) has already developed the "National Surveillance Strategy for AMR in Healthy Food Animals" and the "National Surveillance Strategy for AMR in Diseased Food Animals," followed by the implementation of a national AMR surveillance pilot in healthy food animals. Recognizing the importance of addressing AMR in all food production systems, monitoring in aquaculture and fisheries was deemed equally essential. We are grateful to the Fleming Fund Country Grant Pakistan for taking the lead and providing continuous technical guidance and funding support to initiate and advance AMR-related work in the aquaculture sector. Their support has been instrumental in highlighting the significance of AMR surveillance in fish and aquaculture environments and in shaping the "National Surveillance Strategy for AMR in Aquaculture" and the "Pilot Active AMR Surveillance in healthy Farmed Inland Fish" .The development of this strategy & the pilot study was informed through consultations with provincial fisheries departments, academic institutions, the Aquaculture and Fisheries Program at NARC, the Fisheries Development Board, aquaculture farmers, donor agencies, and other stakeholders, marking an important step toward establishing baseline data and strengthening national capacities.

I congratulate the team for achieving another milestone in the veterinary sector through the support of the Fleming Fund Country Grant, Pakistan. I am hopeful that this momentum will be sustained, and I strongly advocate for continued, long-term efforts to address AMR in the aquaculture sector. This pilot report provides a critical foundation for internal planning and stakeholder dialogue, and should guide the design of future, comprehensive surveillance systems. Strengthening and operationalizing AMR laboratories nationwide, alongside the effective implementation of this strategy, will be critical to safeguarding public health, animal health, and the environment.

Dr. Syed Murtaza Hassan Andrabi
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Message from Team Lead, Fleming Fund Country Grant Pakistan (DAI)

Antimicrobial use (AMU) in the rapidly expanding aquaculture industry may contribute to the rise of antimicrobial resistance (AMR), carrying potential consequences for animal, human, and ecosystem health. Hence, timely detection of antimicrobial-resistant pathogens and continuous monitoring programs are essential. This document will help authorities curb unnecessary antibiotic use and implement appropriate management measures to address this growing threat.

Antimicrobial resistance has become a global public health concern. National governments and international organizations recognize its urgency and are making concerted efforts to curb and control this menace. As the issue is multifaceted and multidimensional, solutions must also be multi-pronged. Moving away from a narrow focus on the human health sector, it is now widely acknowledged that animals and the environment are equally critical and must be integral to all interventions targeting AMR, a principle central to the One Health approach.

The Fleming Fund, established by the United Kingdom in response to the UK AMR Review and the WHO Global Action Plan on AMR, supported Pakistan from 2019 until the completion of the program, contributing significantly to strengthening AMR systems across the human, veterinary, and environmental sectors. As part of this collaboration, the "National Surveillance Strategy for Antimicrobial Resistance in Aquaculture" was developed to guide national efforts toward improved surveillance, antibiotic stewardship, and responsible management practices within the aquaculture sector, an area with important implications for both human and animal health.

With the conclusion of the Fleming Fund program, we hope that the Government of Pakistan will continue to champion and sustain this critical work. Continued investment, commitment, and leadership are essential to maintaining progress in AMR surveillance in the aquaculture sector and ensuring that Pakistan advances its One Health response to AMR.

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Fleming Fund Country Grant Pakistan

Saad Fish Farms & Feed Suppliers, Mansehra

Ghulam Din & Sons Fish Farms Private Limited, Multan

Tawakal Tilapia Hatchery, Muzaffargarh

Pak Gharo Agri & Livestock Farms, Gharo, Thatta

Office of the Director, Fisheries, Fish Biodiversity Hatchery, Chashma

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List of Acronyms

AAHP	Aquatic Animal Health Professionals
AHC	Animal Husbandry Commissioner
AMR	Antimicrobial Resistance
AMR-CU	AMR Coordination Unit
AMU	Antimicrobial Use
ARG	Antimicrobial Resistant Genes
ARB	Antimicrobial Resistant Bacteria
AST	Antimicrobial Susceptibility Testing
CIA	Critically Important Antibiotics
CLSI	Clinical and Laboratory Standards Institute
FFCGP	Fleming Fund Country Grant Pakistan
FPA	Food Producing Animals
HIA	Highly Important Antibiotics
HPCIA	Highest Priority Critically Important Antibiotics
KPK	Khyber Pakhtun Khawa
LMIC	Low- and Middle-Income Countries
MIA	Medically Important Antibiotics
MALDI-ToF	Matrix-Assisted Laser Desorption/Ionization Time-of-Flight
MDR	Multi Drug Resistance
MoNFS&R	Ministry of National Food Security & Research
MS	Mass Spectrometry
NRLPD	National Reference Laboratory for Poultry Diseases
NRL	National Reference Laboratory
NVL	National Veterinary Laboratories
PGA&LF	Pak Gharo Agri & Livestock Farms
SEA	South East Asia
SDG	Sustainable Development Goals
TSI	Triple Sugar Iron
TWG	Technical Working Group
VS	Veterinary Services
WOAH	World Organization of Animal Health
WHO	World Health Organisation

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Executive Summary

This report presents findings from a pilot study representing Pakistan's first Pilot Active AMR Surveillance in Healthy Inland Farmed Fish, which was implemented to establish foundational methods, build capacity, and generate preliminary baseline data. The sector is expanding rapidly and plays an increasingly critical role in national food security, livelihoods, and rural economic development. Despite its growing importance, antimicrobial resistance (AMR) in aquaculture has remained a major surveillance gap. In response, the Fleming Fund Country Grant (FFCG) supported the Ministry of National Food Security & Research (MoNFS&R) in designing and implementing the country's first Pilot Active AMR Surveillance in Healthy Inland Farmed Fish, thereby establishing baseline data, strengthening national technical capacity, and operationalizing a One Health approach in this previously unmonitored sector.

The pilot targeted *Escherichia coli* and *Aeromonas* spp. from three economically important farmed fish species, *Labeo rohita* (rohu), *Oreochromis niloticus* (tilapia), and *Oncorhynchus mykiss* (trout). Different fish species were sourced from nine different sentinel sites across Punjab, Sindh, and Khyber Pakhtunkhwa (KPK) (Table 1). A total of 364 fish samples were collected for the recovery of the two microorganisms followed by antimicrobial susceptibility testing (AST), providing the first evidence base for AMR in Pakistan's inland aquaculture systems.

Key Findings

Findings from this limited pilot indicate that aquaculture systems are exposed to and can harbour antimicrobial-resistant bacteria, with patterns suggesting contamination from broader environmental and anthropogenic sources.

- **Recovery of indicator and aquatic bacteria:** *E. coli* was isolated from 40.2% of rohu, 46.7% of tilapia, and 33.3% of trout, suggesting possible faecal or environmental contamination within aquaculture systems. *Aeromonas* spp., a well-recognized pathogenic microorganism in aquatic animals, was also frequently isolated (46.3% in rohu; 32% in tilapia and 37.8% in trout).
- **High resistance to WHO Highest Priority Critically Important Antimicrobials (HPCIAs):** *E. coli* from rohu and tilapia exhibited very high resistance to ciprofloxacin (up to 65.7%) and moderate to high resistance to third-generation cephalosporins such as cefotaxime (up to 30.6%).
- **Detection of carbapenem resistance:** Notably, 8.6% of *E. coli* from tilapia were resistant to imipenem—an antibiotic reserved for the treatment of severe human infections—indicating potential spillover of highly resistant strains into aquaculture environments.
- **Extremely high resistance to aminopenicillin:** Ampicillin resistance was widespread in *E. coli* across species (up to 84.7% in rohu).
- **Species-specific resistance patterns:** *E. coli* from trout showed markedly lower resistance to several antimicrobials (e.g., chloramphenicol, tetracycline, trimethoprim-sulfamethoxazole), highlighting differences in environmental exposures or selective pressures between cold-water and warm-water systems. *Aeromonas* spp. from trout, however, showed higher resistance to key antimicrobials than Aeromonas from other species.
- **Multidrug resistance (MDR):** MDR (classified as isolates that exhibited resistance to antimicrobials belonging to at least 3 classes) *E. coli* was detected at concerning levels: 48.6% in tilapia, 38.8% in rohu, and 33.3% in trout. While overall MDR in *Aeromonas* spp. was lower, one trout isolate exhibited resistance to six antibiotic classes.

The presence of microorganisms resistant to WHO's HPCIAs, extremely high-level resistance to commonly used antimicrobials such as aminopenicillin, and the occurrence of MDR bacteria in healthy fish intended for human consumption indicates a potential transmission pathway for resistant pathogens and AMR genes (ARGs) into human populations via the interconnected agri-food systems. This underscores the importance of strengthening AMR stewardship in aquaculture production and limiting the self-perpetuating cycle of cross-contamination with AMR organisms in Pakistan's aquaculture systems and the broader environment.

The relatively small sample size, especially for tilapia and trout, restricts the generation of robust national bacterial recovery and AMR estimates; however, this pilot provides essential baseline evidence and a replicable framework including field sampling methodologies and laboratory techniques for future scaling nationwide surveillance in healthy aquatic animals in Pakistan.

1. Introduction

1.1 Background

1.1.1 Global Context

Antimicrobial resistance (AMR) is one of the most urgent global health threats, affecting humans, animals, plants, and ecosystems. In 2019, bacterial AMR was estimated to have caused 1.27 million human deaths worldwide, with the heaviest burden occurring in low- and middle-income countries (LMICs), particularly in sub-Saharan Africa and South Asia[1]. Without effective interventions, annual global deaths attributed to AMR are projected to reach 10 million by 2050[2]. Beyond its direct impact on health, AMR undermines progress toward multiple Sustainable Development Goals (SDGs), particularly those related to poverty reduction, food security, health, and economic stability. Antimicrobials are widely used in food-producing animals for both prophylactic and therapeutic purposes. In response to the increasing global demand for meat, production is projected to rise from 200 million tons to 470 million tons by 2050[3]. As livestock production expands, farmers increasingly rely on antimicrobials to treat and control diseases in animals. The use of antimicrobials in food animals has raised significant concerns, as it is associated with the rise, emergence, and spread of AMR organisms, particularly those with public health implications, highlighting AMR as a critical One Health issue. Many human cases of AMR have been traced back to AMR microbes originating from livestock[2,3]. The consumption of contaminated meat, along with direct contact with infected animals or their environment, are key pathways through which AMR bacteria are transmitted from livestock to humans[2].

Water and its resources are known to be crucial reservoirs of ARB. Specifically, the misuse of antibiotics in the aquaculture sector plays a crucial role in AMR dissemination globally and, in documented cases, can lead to dramatic consequences to human, animal, and ecosystem health[3]. ARGs in bacteria present in aquatic foods have been previously documented to transmit them along the food chain. A recent study in the Netherlands estimated seafood as the most common food source of *Escherichia coli* containing β-lactam resistance genes[4]. As the ever-increasing human population relies more heavily on aquaculture commodities for food security, the spread of AMR and associated infections through seafood represents a major concern for public health. Despite the key role of the aquaculture supply chain and its inclusion within the One Health framework, little to no implementation in controlling the spread of AMR has occurred in global aquaculture[5].

For aquatic diseases in Southeast Asia resistance to 17 antimicrobial classes has been reported[6]. According to this study by Bongkotrat et al., (2023), resistance to the following classes was frequently observed: aminoglycosides, beta-lactams, (fluoro) quinolones, tetracyclines, and sulphonamides. Additionally, beta-lactams, tetracyclines, as well as sulphonamides were observed at levels above 40% in isolates tested. In terms of antimicrobial resistant bacteria (ARB), the indicator Gram-negative organism, *Escherichia coli,* and foodborne pathogens *Aeromonas* spp. and *Vibrio* spp. were the most widely and frequently reported ARB in the South East Asia (SEA) aquaculture sector.

The need for intervention to control AMR in aquaculture is evidenced by the abundant scientific literature that reveals high levels of AMR in aquaculture environments and the associated risks to human, animal, and environmental health, especially in LMICs[3,5,6]. Moreover, as the pressure on food security and climate change intensifies, AMR in aquaculture would continue to increase, considering that indiscriminate use of antimicrobials is common in many countries, including various top aquatic animal producing countries[7]. The literature reported diverse use of antimicrobial active ingredients, where an average of 15 antimicrobials per country are used in the top 15

aquaculture producing countries, of which more than half fall in the category of LMIC[8]. It is also predicted that AMR in aquaculture would primarily impact countries with higher temperatures[7].

An essential measure to minimise the risk of AMR in aquaculture is to monitor AMU at a national, regional, and global level and advocate AMU reduction through better stewardship among involving farmers, veterinarians/aquatic animal health professionals (AAHP) and Veterinary Services (VS)/Aquatic Animal Health Services (AAHS). However, in aquaculture, monitoring AMU is a complicated task due to the diversity of species and culture systems, the unconsolidated nature of production in many regions, and the commonly unregulated use of antimicrobials, for example, over-the-counter use and off-label use of antimicrobials[9]. Over 90% of the world aquaculture production is carried out in LMICs, where regulation and enforcement, as well as practices and resources in aquaculture, are limited. Furthermore, often antimicrobial treatments are administered without professional consultation or uninformed by any susceptibility testing[10]. Due to the high costs of developing new antibiotic molecules, antibiotic agents used in the human and veterinary sectors are also used in the aquaculture sector. Six common classes of antibiotics (aminoglycosides, macrolides, aminopenicillin, quinolones, sulphonamides, and tetracyclines) that are regularly used in aquaculture and in livestock are listed by the World Health Organization (WHO) as critically or highly important antimicrobials[9,11,12].

Aquatic environment has been increasingly shown to be a reservoir for ARB. Isolation of multiple bacterial species from aquaculture and seafood products in Thailand, including *Aeromonas* species, *Vibrio cholerae*, and *Enterococcus faecium* recovered from market fish and shrimp, suggests the significance of the aquatic environment as an AMR reservoir[13]. These isolates exhibited notable AMR, particularly to Critically Important Antibiotics (CIA) such as tetracyclines, fluoroquinolones, and β-lactams. The presence of MDR species, especially Aeromonas spp., which are common aquatic pathogens with zoonotic potential, highlights Thailand's aquaculture sector as an important reservoir of AMR bacteria. Overall, the findings demonstrate significant public health concerns, emphasizing the need for strengthened surveillance and prudent antibiotic use in aquaculture.

External sources of AMR such as livestock and human wastewater that affect aquaculture environments are also critical and demand rigorous investigation. In LMICs, combining livestock and aquaculture in integrated farming systems presents an option for increased productivity, yet the potential exchange of ARBs and their genes from livestock wastes increases the risk of AMR in those systems[8]. Such a combination of unregulated drug use, intensive fish production and unchecked circulation of ARBs from aquatic animals to humans has created a perfect AMR storm that could lead to serious and long-term consequences. Country and regional programs for the surveillance and monitoring of AMR in bacteria isolated from aquatic animals are necessary, as described in the World Organization of Animal Health (WOAH)'s Aquatic Code[1,14,15].

1.1.2 Strategic Importance and the AMR Challenge in Pakistan's Aquaculture

Pakistan's fisheries sector is a strategic national asset, contributing to food security, livelihoods, and export earnings according to Economic Survey of Pakistan (2024-2025). While it constitutes 0.31% of gross domestic product (GDP), its value extends beyond economic metrics. As a vital sub-sector of agriculture, it provides a crucial alternative protein source and sustains the incomes of coastal and inland freshwater communities. Production for year 2024 (July-April) reached 720.9 thousand metric tons (MT), with a significant 310 thousand MT originating from inland waters. This substantial inland output, driven primarily by aquaculture, highlights the sector's growth potential and underscores the critical need for sustainable development and risk management.

A primary risk stems from the intensification of freshwater fish farming, where the potential for antimicrobial use (AMU) to manage disease in dense populations is high. Unchecked AMU can lead

to AMR, creating resistant pathogens that threaten animal health, compromise food safety for consumers, and facilitate the environmental spread of AMR genes. Consequently, studying the emergence and spread of AMR within this system is vital. Addressing this threat is essential to safeguard both the sector's domestic food security role and its impressive export trajectory, which saw earnings of USD 534.2 million in 2024 (July-March)[16]. Integrating AMR surveillance and stewardship is therefore fundamental to the future of sustainable aquaculture in Pakistan.

1.1.3 Addressing a National Surveillance Gap

Despite the sector's importance, systematic national surveillance for AMR in aquaculture remained a significant, documented gap. A 2024 peer-reviewed assessment of Pakistan's first National Action Plan on AMR (2017-2022) revealed that interventions in agriculture were largely at the "planning and piloting stages," with "insufficient attention to non-livestock sectors" like aquaculture[17]. This finding aligned with the Ministry of National Food Security & Research's (MoNFS&R) own acknowledgement that "AMR data from aquaculture production in Pakistan is a knowledge gap" in understanding pathogen transmission across the One Health spectrum[18].

The imperative to establish a national surveillance strategy was therefore twofold. First, it was a direct response to an identified policy implementation shortfall, aiming to move beyond planning to actionable systems. Second, it addressed a critical data vacuum; the absence of a coordinated system made it impossible to establish baseline resistance levels, track trends, or inform evidence-based policies for this vital sector.

1.2 AMR Surveillance in Aquaculture

In January 2024, the launch of the Fleming Fund Country Grant in Pakistan (FFCGP)-Phase II provided a pivotal opportunity to address this gap. With an explicit aim to deepen the integration of a One Health approach into Pakistan's animal health AMR surveillance, the FFCGP partnered with the MoNFS&R to strategically extend the national framework to include aquaculture. This collaboration was supported by FFCGP's technical and financial assistance in formulating a 'National AMR Surveillance Strategy in Aquaculture'.

As a cornerstone of this strategy, FFCGP spearheaded the design and launch of a 'Pilot Active AMR Surveillance in Healthy Inland Farmed Fish'. This pilot was conceived to directly address the heightened AMU risk driven by disease outbreaks in Pakistan's prevalent semi-intensive farming systems. Its purpose was to establish a practical proof-of-concept, demonstrating the feasibility and value of systematic surveillance in this food production chain. This report presents the methodology, findings, and implications from that pioneering 2024-2025 pilot program.

To anchor this initiative within a robust national framework, the MoNFS&R took concrete institutional steps. The National Veterinary Laboratory (NVL) was formally appointed as the designated National Reference Laboratory (NRL) for aquaculture. Operational leadership for data collection, collation, and analysis was assigned to the Ministry's AMR-Coordination Unit (AMR-CU).

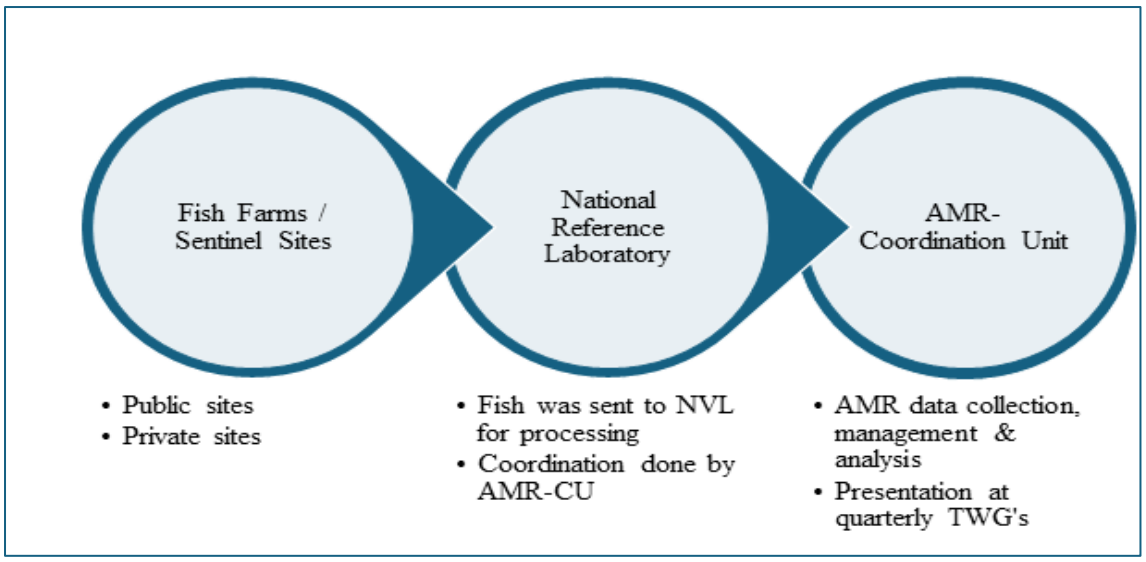

Figure 1: Flow of activities in active AMR surveillance in fish

Furthermore, FFCGP championed a transformative public-private alliance by facilitating a Memorandum of Understanding (MoU) between private aquaculture sentinel sites and DAI, the FFCGP implementation lead in Pakistan, with the MoNFS&R officially witnessing the agreement. This partnership is a critical step towards a coordinated sector-wide approach to AMR surveillance.

This surveillance initiative operates within a distinct and evolving sectoral context. Pakistan possesses an extensive, underutilized freshwater network of rivers, lakes, and canals, representing significant growth potential. Currently, aquaculture remains relatively nascent, with private-sector investment driving the establishment of dug-out pond farms utilizing semi-intensive practices as the prevailing model. Production is dominated by premium-priced carps, particularly rohu, while the commercial farming of species like catfish, snakeheads, and tilapia is still in a developmental phase. It is within this dynamic landscape that the foundational work of AMR surveillance has been initiated[19].

1.2.1 Aims and objectives

The aim of the study was to initiate and integrate AMR surveillance within Pakistan's aquaculture sector, in alignment with the National Action Plan on AMR. Specifically, the study sought to establish foundational systems, capacities, and evidence needed to support long-term integrated One Health AMR monitoring in healthy aquatic animals (inland fish).

The objectives were two-fold

- To strengthen national capacity across both public and private sector aquaculture stakeholders for implementing AMR surveillance, including standardized sampling, laboratory isolation and AST, and data reporting and communication.
- To characterize and compare the AMR phenotypes of the indicator organism, *E. coli*, and pathogenic bacteria *Aeromonas* spp. recovered from rohu, tilapia, and trout, including the prevalence of multidrug resistance (MDR) and resistance to WHO's medically important antimicrobials (MIA) for human medicine.

2. Methodology

2.1 Sampling sites and target fish species

Under the auspices of the MoNFS&R, the FFCGP Phase II successfully established a network of nine sentinel sites dedicated to active AMR surveillance within Pakistan's aquaculture sector. This network, illustrated in the map below, comprised a blend of five private and four public facilities strategically located across three major fish-producing provinces: Punjab, Khyber Pakhtunkhwa (KPK), and Sindh.

From these sentinel sites, samples were submitted to the designated NRL, the National Veterinary Laboratory (NVL), for bacterial isolation and to perform AST. The NVL collated AMR data using a pre-designated format and forwarded it to the AMR-CU at the MoNFS&R. The AMR-CU analysed the data and presented its findings during quarterly meetings of the Technical Working Group (TWG).

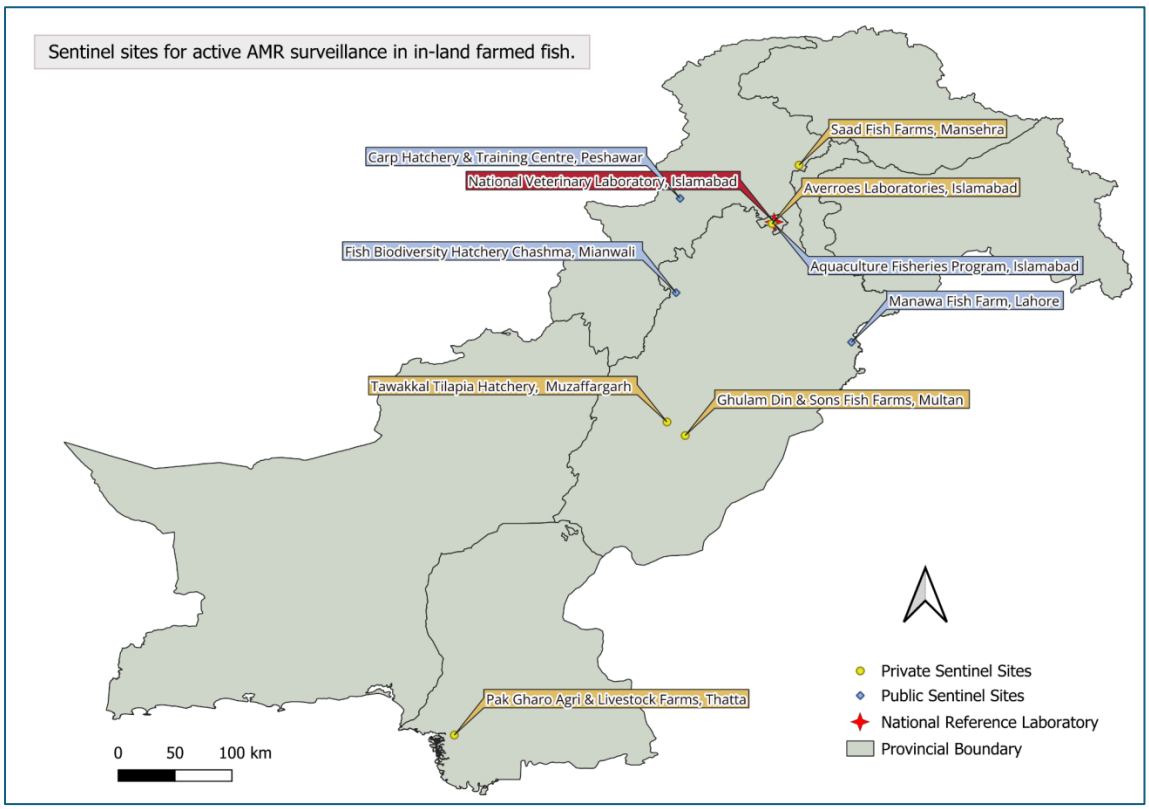

Figure 2: Sentinel sites for active AMR surveillance in inland farmed fish.

The pilot study focused on three fish species of paramount economic importance to Pakistan: *Oreochromis niloticus* (tilapia), *Labeo rohita* (rohu), and *Oncorhynchus mykiss* (trout). Tilapia samples were procured primarily from Pak Gharo Agri & Livestock Farms (PGA&LF) in Sindh, with supplementary contributions from Tawakal Tilapia Hatchery, Averroes Laboratories, and the Aquaculture Fisheries Program in Punjab. Trout samples were sourced exclusively from Saad Fish Farms in Khyber Pakhtunkhwa (KPK). The remaining sentinel sites in KPK and Punjab submitted rohu, which emerged as the most sampled species across the network. It is important to note that the diversity of available fish stock, coupled with the seasonal nature of harvesting cycles in Pakistan, precluded the consistent collection of identical species over an extended period (Table 1).

Table 1: Sentinel sites on-boarded for active AMR surveillance in aquaculture and the fish species sent by each site.

Site #	Site Name	Fish species		
		Trout (Tr)	Rohu (R)	Tilapia (Ti)
1.	Saad Fish Farms & Feed Suppliers, Mansehra	X		
2.	Ghulam Din & Sons Fish Farms Pvt. Limited, Multan		X	
3.	Tawakal Tilapia Hatchery, Muzaffargarh		X	X
4.	Pak Gharo Agri & Livestock Farms, Gharo, Thatta			X
5.	Averroes Laboratories, Islamabad		X	X
6.	Fish Biodiversity Hatchery, Chashma		X	
7.	Carp Hatchery & Training Centre, Peshawar		X	
8.	Fisheries Research and Training Institute, Manawa Fish Farm, Lahore		X	
9.	Aquaculture & Fisheries Program, NARC, Islamabad		X	X

2.2 Sampling strategy

The fish sampling commenced in late October-November, following the procurement of necessary consumables and equipment. Each month, the sentinel sites aimed to submit five (05) healthy fish samples per batch to NVL through the facilitation provided by FFCG and AMR-CU. Though not all sites were able to submit five samples regularly. The details of fish sampling over the nine month period with counts of fish submitted by each site are present in Table 2.

Table 2: Batch wise fish sampling was done over a period of nine months (Nov-July). Of the nine sites, three submitted tilapia sporadically, while PGA&LF submitted tilapia all nine months; Saad fish farm submitted trout; rohu was submitted by six sites of which two submitted sporadically, while four sent samples all nine months. The counts of fish submitted by each site was maximum five. A total of 75 tilapia, 45 trout and 244 rohu were received by NVL.

Fish species	Site	Nov	Dec	Jan	Feb	March	April	May	June	July	Total per site	Total per species
Tilapia	Tawakal Tilapia Hatchery, Muzaffargarh	5	5	5							15	75
	Pak Gharo Agri & Livestock Farms (PGA&LF), Gharo, Thatta	5	5	5	5	5	5	5	5	5	45	
	Averroes Laboraties, Islamabad					5					5	
	Aquaculture & Fisheries Program, NARC, Islamabad		5	5							10	
Trout	Saad Fish Farms & Feed Suppliers, Mansehra	5	5	5	5	5	5	5	5	5	45	45
Rohu	Ghulam Din & Sons Fish Farms Pvt. Limited, Multan	5	5	6	5	4	5	5	5	5	45	244
	Tawakal Tilapia Hatchery,	5	5	5	5	5	5	5	5	5	45	

	Muzaffargarh											
	Office of the Director Fisheries, Fish Biodiversity Hatchery, Chashma	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**45**	
	Carp Hatchery & Training Centre, Peshawar	**5**	**5**	**0**	**4**	**5**	**4**	**6**	**5**	**6**	**40**	
	Quality Control Labs, Fisheries Research and Training Institute, Manawa Fish Farm, Lahore		**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**40**	
	Averroes Laboraties, Islamabad			**5**	**5**		**5**	**4**	**5**	**5**	**29**	
	Total per batch	**35**	**45**	**46**	**39**	**39**	**39**	**40**	**40**	**41**		**364**

The sample size and distribution were determined by the operational scope of this initial pilot study. Consequently, the sample is not statistically representative at a provincial or national level, and the results should be interpreted as indicative rather than definitive.

2.2.1 Fish collection and transportation

The sampling protocol entailed random selection of five fish each from five distinct ponds within each farm. The selected fish were then individually weighed, wrapped, labelled, and sealed before being placed in an ice-filled ice box to prevent spoilage during transportation. The sentinel sites also completed a 'Farm Data Collection Form' which accompanied the fish samples to NVL.

Figure 3: Images of sample packing and transportation at different sentinel sites. Left: Fish is being weighed before packaging at Tawakal Tilapia Hatchery. Centre: Labelled fish is packed in icebox at Saad Fish Farm. Right: Fish being placed in iceboxes at PGA&LF.

To ensure the integrity of the fish samples, transportation to NVL was carried out within 8-12 hours post-of removal from water, thereby preventing putrefaction and facilitating successful microbiological isolation. Upon receipt at NVL, a post-mortem examination was conducted, and the intestines were removed for bacterial isolation using intestinal contents. A total of 364 fish samples were sent to NVL. Of these 244 were rohu, 75 were tilapia and 45 were trout. During peak winter season, some sites were unable to net the fish to avoid stress-induced mortality (Table 3).

Table 3: Total fish count per species collected over a period of nine months from different sentinel sites.

Fish species	Count	Total
Rohu	244	364
Tilapia	75	
Trout	45	

2.2.2 Fish Post-mortem for sample collection

Upon receipt, the fish were prepared for post-mortem examination under stringent aseptic conditions to procure intestinal contents for subsequent bacterial isolation and identification. Prior to necropsy, the external surface of each specimen was thoroughly rinsed with sterile distilled water to eliminate extraneous debris and potential surface contaminants. Each fish was then positioned on a sterile dissecting tray, with standard personal protective equipment (including gloves and a laboratory coat) utilised throughout the procedure.

A longitudinal incision was made with sterile instruments along the ventral midline, extending from the anal opening to the operculum. The abdominal cavity was subsequently opened with care to expose the gastrointestinal tract. Following identification, the intestines were gently exteriorised to avoid rupturing the lumen. A segment of the intestine was isolated using sterile forceps and scissors, and then opened longitudinally to access the intestinal contents.

Faecal material was collected aseptically from the lumen using sterile swabs or spatulas and was immediately transferred into sterile containers. For enrichment purposes, samples were placed into pre-labelled tubes containing buffered peptone water. Throughout laboratory processing, all samples were maintained on ice to preserve bacterial viability and were processed within a few hours of collection to ensure optimal results.

2.3 Microbiological isolation

The pilot AMR surveillance targeted two bacteria - *Escherichia coli* and *Aeromonas* spp. The inclusion of *E. coli* and *Aeromonas* spp. aligns with international recommendations for AMR testing in aquatic animals. *E. coli* serves as a standardised, WHO-recommended indicator for faecal contamination and the potential dissemination of relevant AGRs from human and animal reservoirs into the environment[20,21]. Concurrently, *Aeromonas* spp. are native aquatic pathogens of direct veterinary and emerging public health concern, intrinsically exposed to selective pressures in aquaculture settings, making them critical sentinels for resistance development within the sector [22,23].

2.3.1 Isolation and Identification of *E. coli*

The samples were first enriched in buffered peptone water (Oxoid, Hampshire, UK) and incubated for 24 hours at 37°C to allow recovery and proliferation of stressed or low-level bacterial cells. Following enrichment, aliquots were streaked onto MacConkey agar (Oxoid, Hampshire, UK) and incubated for 24 hours at 37°C. A single well-isolated colony from each plate, exhibiting typical *E. coli* morphology (smooth, circular, pink lactose-fermenting colonies), was selected as a presumptive *E. coli* isolate. The selected colonies were further sub-cultured on fresh MacConkey agar to ensure purity.

Pure cultures were subjected to conventional biochemical testing. The diagnostic panel included indole, methyl red, Voges–Proskauer, citrate utilization, and triple sugar iron (TSI) tests. All culture media and reagents used for biochemical identification were commercially obtained from Oxoid (Hampshire, UK). Isolates demonstrating indole positivity, methyl red positivity, and typical TSI

reactions (acid/acid with gas production), along with negative results for Voges–Proskauer and citrate utilization, were classified as *E. coli*.

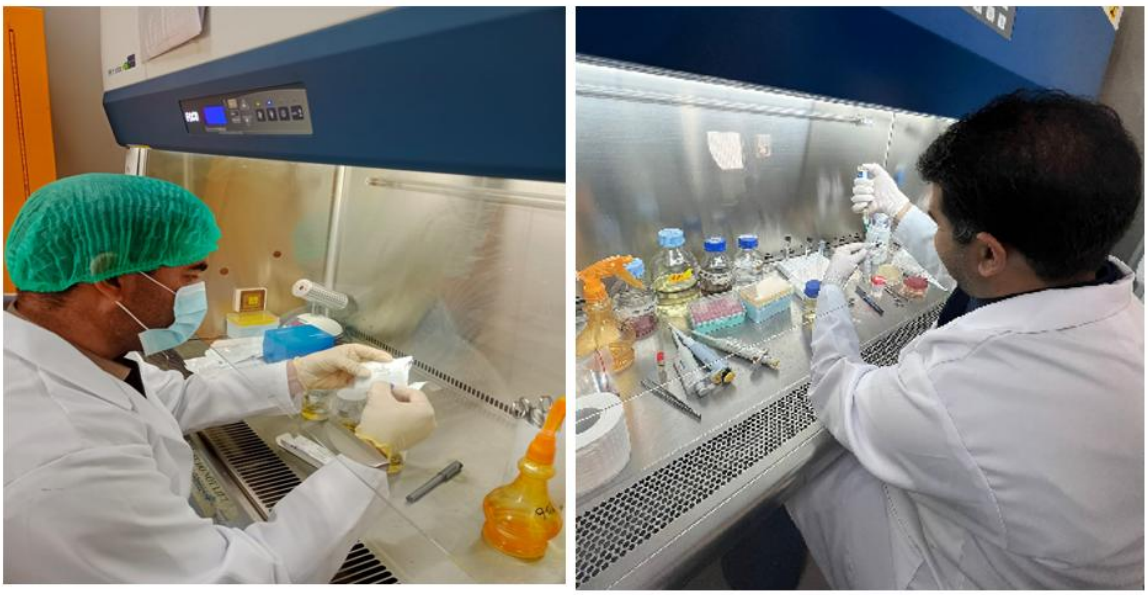

Figure 4: Microbiological processing of samples from fish.

Presumptive *E. coli* isolates were further confirmed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF MS). For MALDI-ToF analysis, a small portion of a fresh colony was applied to a target plate and overlaid with α-cyano-4-hydroxycinnamic acid (HCCA) matrix solution. After air drying, the target plate was introduced into the MALDI-ToF instrument, where laser excitation resulted in ionization of bacterial proteins. The generated mass spectral profiles were obtained in linear positive-ion mode and matched against the manufacturer-provided reference database for microbial identification. Isolates with high-confidence score values (typically ≥ 2.0) were confirmed as *E. coli*.

2.3.2 Isolation and Identification of *Aeromonas* Species

For *Aeromonas* isolation, the samples were first enriched in buffered peptone water (Oxoid, Hampshire, UK) and incubated at 37°C for 24 hours. After enrichment, the samples were streaked onto *Aeromonas* agar (Liofilchem, Italy) supplemented with ampicillin (Liofilchem, Italy). Colonies exhibiting characteristic *Aeromonas* morphology (typically round, smooth, convex, and opaque with a yellowish to greenish hue) were selected as presumptive isolates. One colony per sample was sub-cultured onto fresh Aeromonas agar to obtain pure cultures.

Pure isolates were subjected to a panel of biochemical tests for preliminary identification. The tests included oxidase, indole, motility, and glucose fermentation, using commercially available reagents and media (Oxoid, Hampshire, UK). Isolates showing oxidase positivity, catalase positivity, glucose fermentation, motility, and typical TSI reactions were considered presumptive *Aeromonas* species.
For further confirmation, presumptive *Aeromonas* isolates were subjected to matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF MS). Fresh colonies were transferred onto a MALDI target plate and overlaid with α-cyano-4-hydroxycinnamic acid (HCCA) matrix solution. After air drying, the target plate was inserted into the MALDI-ToF instrument. Laser excitation generated ionized bacterial proteins, primarily ribosomal proteins, which were analysed to produce characteristic mass spectra. The spectra were compared against a reference database and isolates with high-confidence scores were confirmed as *Aeromonas* species.

2.3.3 Antimicrobial Susceptibility Testing

E. coli and *Aeromonas* isolates were subjected to AST using the Kirby-Bauer disc diffusion test following the recommendations of the Clinical Laboratory Standard Institute (CLSI). A panel of antibiotics from different antibiotic classes was used to test susceptibility of *E. coli* and *Aeromonas* isolates (Table 4, Table 5). These antibiotics were selected due to their clinical relevance in veterinary and human health[24].

Table 4: Antibiotics panel with their disc potency and zone diameters for conducting AST against E. coli isolates in fish (CLSI M100, 35th Edition, 2025).

Antibiotics	Potency (µg)	Susceptible (mm)	Intermediate (mm)	Resistant (mm)
Ampicillin	10	≥17	14-16	≤13
Gentamicin	10	≥18	15-17	≤14
Azithromycin	15	≥13	-	≤12
Cefotaxime	30	≥26	23-25	≤22
Ceftazidime	30	≥21	18-20	≤17
Ciprofloxacin	5	≥26	22-25	≤21
Tetracycline	30	≥15	12-14	≤11
Trimethoprim-sulfamethoxazole	250	≥16	11-15	≤10
Nalidixic Acid	30	≥19	14-18	≤13
Chloramphenicol	30	≥18	13-17	≤12
Imipenem	10	≥23	20-22	≤19
Cefepime	30	≥25	-	≤18

Table 5: Antibiotics panel with their disc potency and zone diameters for conducting AST against Aeromonas spp. isolates in fish (CLSI M45, 3rd Edition, 2015).

Antibiotics	Potency (µg)	Susceptible (mm)	Intermediate (mm)	Resistant (mm)
Gentamicin	10	≥15	13-14	≤12
Cefotaxime	30	≥26	23-25	≤22
Ceftazidime	30	≥21	18-20	≤17
Ciprofloxacin	5	≥21	16-20	≤15
Tetracycline	30	≥15	12-14	≤11
Trimethoprim-sulfamethoxazole	1.25/23.75	≥16	11-15	≤10
Chloramphenicol	30	≥18	13-17	≤12

Briefly, 2-3 well-isolated colonies from the identified pure isolates were emulsified into 5 mL of sterile normal saline solution, and the turbidity of the solution was adjusted to that of a 0.5 McFarland standard. A sterile cotton swab was immersed in normal saline suspension and uniformly streaked onto a Mueller-Hinton agar medium (Oxoid, UK) plate. Following this, antibiotic discs (Oxoid, UK) containing a specific concentration of antibiotics were placed on the agar surface, and the plates were incubated at 37°C for 24 hours. The interpretation of the zone of inhibition was carried out following the guidelines of CLSI (M45, 3rd Edition)[25] for *Aeromonas* spp. and CLSI M100 (35th Edition)[26] for *E. coli*.

2.4 Statistical Analysis

For each fish species, the isolation percentage was calculated separately for *E. coli* and *Aeromonas* spp.

The AST data was interpreted using CLSI clinical breakpoints and classified isolates as resistant, Intermediate and susceptible (RIS). A descriptive analysis was then conducted to characterise the AMR phenotypes in rohu, tilapia, and trout. For the calculation of non-susceptible percentage in this surveillance program, isolates exhibiting intermediate susceptibility were grouped with resistant isolates. This aligns with the standard methodology for AMR surveillance, which reports the percentage of non-susceptible isolates to provide a conservative and public health-relevant estimate. Therefore, RIS results were transformed into binary outcome measures (0-susceptible and 1-non-susceptible (resistant/intermediate))[26]. The resulting non-susceptible/resistance profiles were contextualised with reference to the WHO priority list of MIA - Critically Important Antibiotics (CIA), Highly Important Antibiotics (HIA), and Highest Priority Critically Important Antibiotics (HPCIA)[27].

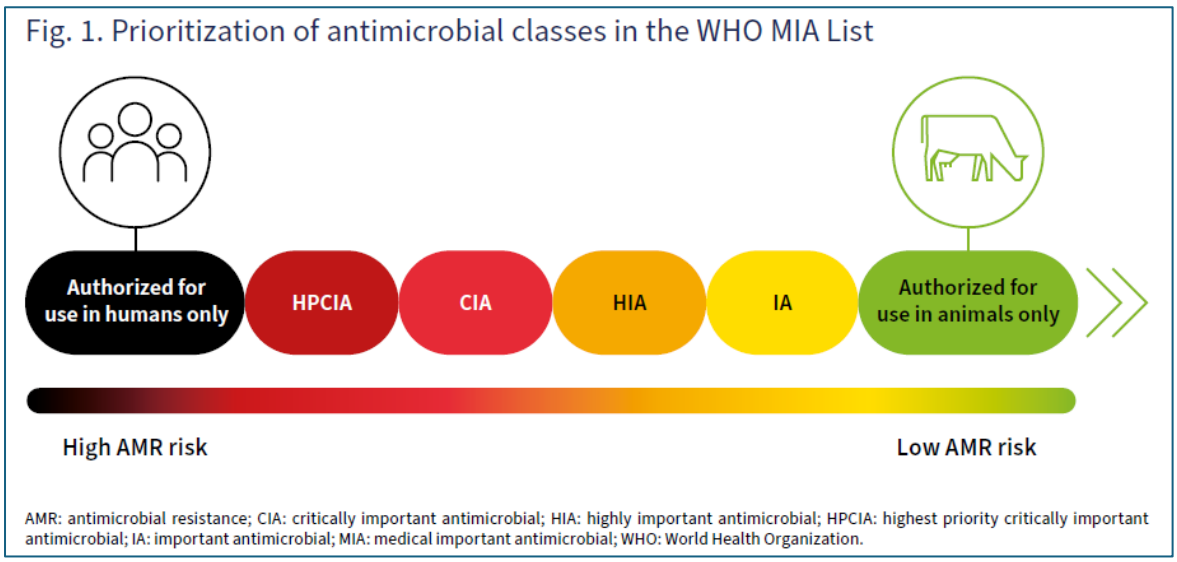

Figure 5: Adapted from WHO's List of MIA: a risk management tool for mitigating AMR due to non-human use. Geneva: World Health Organization; 2024. Licence: CC BY-NC-SA 3.0 IGO. Figure 1. Pg#04[27].

To visualise these profiles, heat maps incorporating hierarchical clustering were generated, employing resistance categories defined by the European Food Safety Authority in the European Union Summary Report. The resistant categories range from 'rare' (<0.1%), 'very low' (0.1%–1.0%), 'low' (>1%–10.0%), 'moderate' (>10.0%–20.0%), 'high' (>20.0%–50.0%), 'very high' (>50.0%–70.0%), to 'extremely high' (>70.0%)[28].

Multi-class resistance profiles, including MDR profiles were determined for each bacterial species. The MDR prevalence in *E. coli* and *Aeromonas* isolates was compared across fish species using contingency-table statistical methods. The percentage of each unique AMR resistance phenotype was obtained.

For all calculated proportions, a binomial proportion confidence interval was determined using the Wilson method, selected for its reliable coverage with the smaller sample sizes present in some groups.

For inferential statistics, chi-square tests were applied to test specific hypotheses. The primary null hypothesis stated that the distribution of antibiotic resistance does not differ significantly among the three fish species for *Aeromonas* spp. isolates. A secondary analysis compared MDR isolates against fully susceptible samples to identify significant differences in their resistance patterns. Fisher's exact test was used instead of the chi-square test where frequency counts were less than 05. The results were not significant and will not be discussed further.

All data were compiled, cleaned and analysed in Microsoft Excel software (Microsoft 365) while the analyses were concurrently performed using the R programming language, version 4.5.1. For visualisation of data R V.4.5.1 was used.

3. Results

Bacterial recovery from fish samples was conducted from late November 2024 through July 2025. Over this period, a total of 364 fish samples were submitted to the NVL for microbiological isolation and AST. The samples included 45 trout, 75 tilapia, and 244 rohu. All sampled fish were in their active growth phase as commercial harvesting typically occurs from November to February. To maintain continuous production, farms routinely manage multiple ponds containing fry of different ages. The average weights of rohu, tilapia and trout examined in the study were 818.7g, 320.2g and 258.9g, respectively.

3.1 Bacterial Isolation Percentage

Figure 6 illustrates the recovery of *E. coli* from healthy rohu, tilapia and trout. Of the 244 rohu samples, 98 tested positives for *E. coli*, yielding an isolation percentage of 40.2% (95% CI: 34.2–46.4). In tilapia, 35 of 75 samples were positive, corresponding to an isolation percentage of 46.7% (95% CI: 35.8–57.8). Among the 45 trout samples, *E. coli* was detected in 15, resulting in an isolation percentage of 33.3% (95% CI: 21.4–47.9).

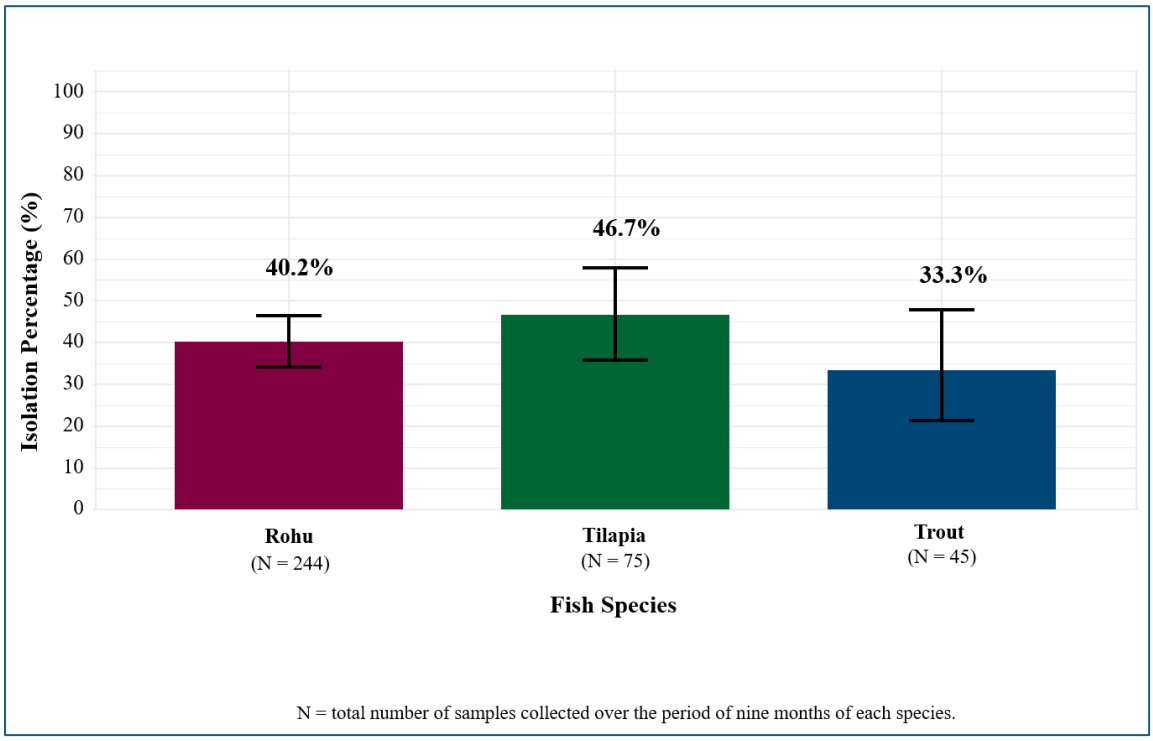

Figure 6: Bar graph with 95% confidence intervals showing isolation percentage of E. coli from different fish species.

Rohu yielded the highest number of *E. coli* isolates tested (n = 98) owing to a larger sample size, followed by tilapia with approximately one-third that number (n = 35), and finally trout with the fewest isolates (n = 15).

Table 6: Isolation percentage of E. coli from different fish species along with 95% confidence intervals.

Fish species	Total samples (N)	*E. coli* positive	Isolation (%)	95% CI
Rohu	244	98	40.2	34.2 - 46.4
Tilapia	75	35	46.7	35.8 - 57.8
Trout	45	15	33.3	21.4 - 47.9

Figure 7 shows the isolation percentage of *Aeromonas* spp. from healthy rohu, tilapia and trout. From a total of 244 rohu samples, 113 were positive for *Aeromonas* spp., yielding an isolation percentage of 46.3% (95% CI: 40.2-52.6). Among the 75 tilapia samples, 24 were positive, corresponding to an isolation percentage of 32% (95% CI: 22.5–43.2). From the 45 trout samples, 17 were positive, resulting in an isolation percentage of 37.8% (95% CI: 25.1–52.4).

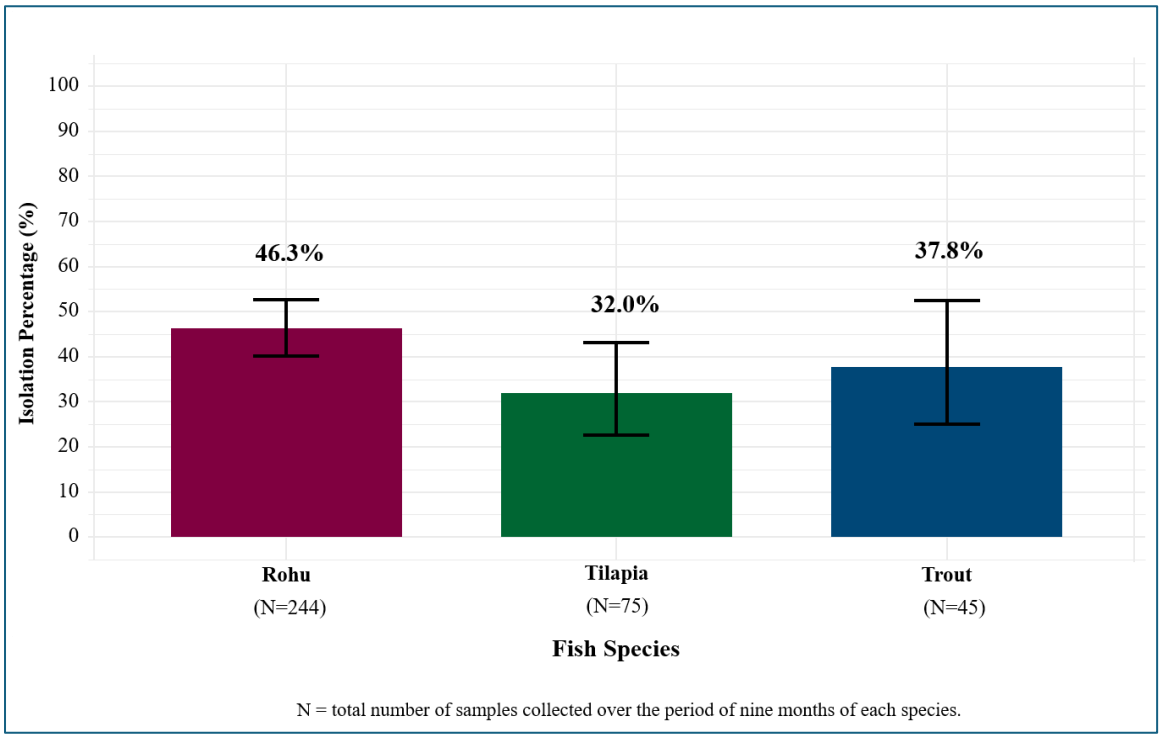

Figure 7: Bar graph with 95% confidence intervals showing isolation percentage of Aeromonas spp. from different fish species.

Rohu yielded the highest number of *Aeromonas* spp. isolates tested (n = 113) owing to a larger sample size, followed by tilapia with approximately one-fourth that number (n = 24), and finally trout with the fewest isolates (n = 17).

Table 7: Isolation percentage of Aeromonas spp. from different fish species along with 95% confidence intervals.

Fish species	Total samples (N)	*Aeromonas* Positive	Isolation (%)	95% CI
Rohu	244	113	46.3	40.2 - 52.6
Tilapia	75	24	32	22.5 - 43.2
Trout	45	17	37.8	25.1 - 52.4

3.2 AMR Phenotypes in Fish

3.2.1 Heat map analysis of antimicrobial susceptibility patterns of bacterial isolates in fish

The AST phenotype of both *E. coli* and *Aeromonas* spp. isolates was assessed. Based on the matrix of resistance percentages, a heat map was generated employing the categorisation framework of the EUSR. This visualisation translated the numerical resistance data into a colour gradient

corresponding to the predefined categories ranging from 'Rare' (Dark blue) to 'Extremely High' (Red). Hierarchical clustering was applied to both the antibiotics (rows) and the fish species (columns). The resulting heat map reveals distinct patterns (Figure 8, Figure 9).

The heat map in Figure 8 depicts the resistance profile of *E. coli* to the twelve antibiotics in the AST panel. The resistance patterns observed for rohu and tilapia were notably similar. For rohu, ten of the twelve antibiotics (tetracycline, trimethoprim-sulfamethoxazole, nalidixic acid, ciprofloxacin, ampicillin, azithromycin, cefepime, cefotaxime, ceftazidime, gentamicin) exhibited resistance categorised as moderate to extremely high, while for tilapia, eight of twelve antibiotics (tetracycline, trimethoprim-sulfamethoxazole, nalidixic acid, ciprofloxacin, ampicillin, azithromycin, cefepime, cefotaxime) fell within this range. In contrast, trout presented a distinct resistance profile, with only three (ciprofloxacin, ampicillin, cefotaxime, gentamicin) of the twelve antibiotics demonstrating moderate to extremely high resistance. The detail counts of each cell in the heat map are presented in Annex I - Table 9.

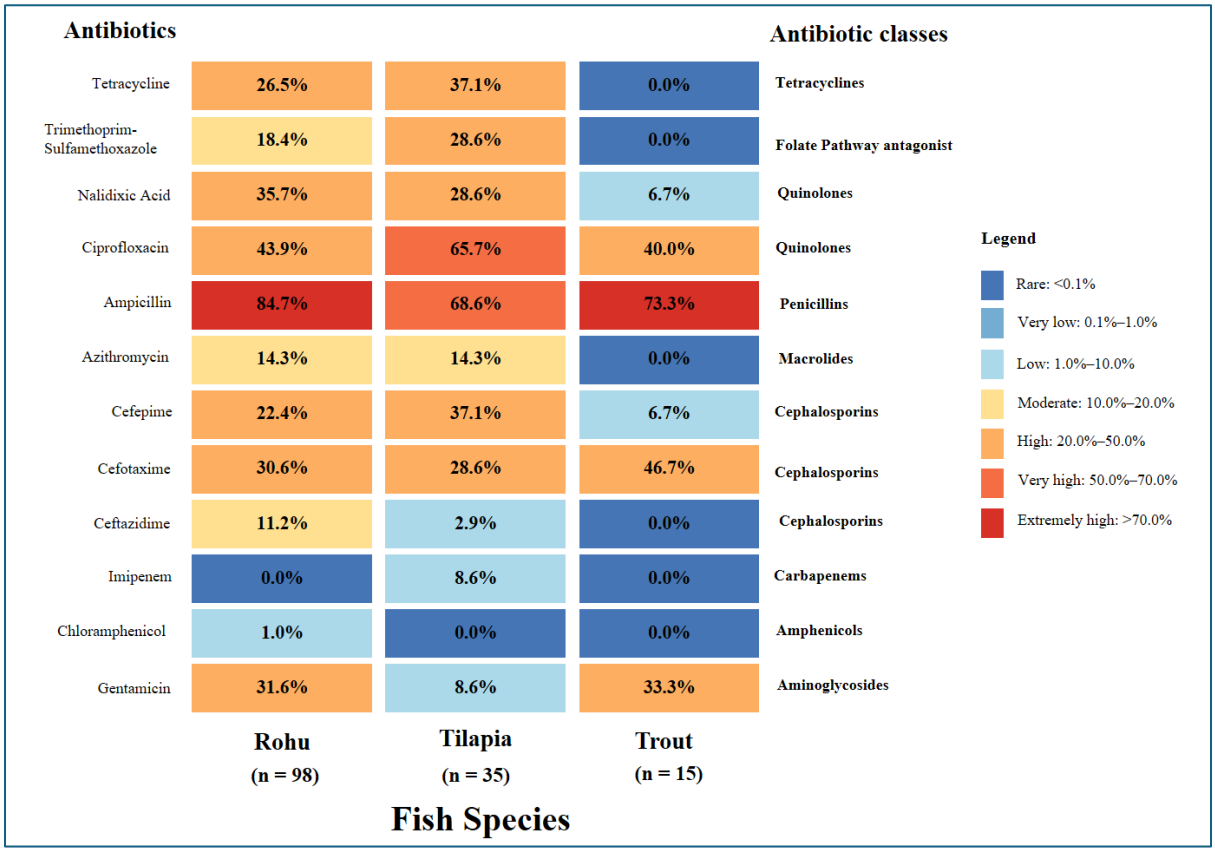

Figure 8: Heat map of resistance percentages in E. coli isolated in rohu, tilapia and trout. The legend uses the EUSR's categorisation system.

The heat map in Figure 9 depicts the resistance profile of *Aeromonas* spp. to seven antibiotics in the AST panel. The resistance patterns observed for rohu and tilapia were notably similar again. For rohu, six of the seven antibiotics (tetracycline, trimethoprim-sulfamethoxazole, ciprofloxacin, ceftazidime, chloramphenicol, gentamicin) exhibited resistance categorised as low to very low, while for tilapia, five of the seven antibiotics (tetracycline, trimethoprim-sulfamethoxazole, ciprofloxacin, ceftazidime, chloramphenicol) fell between rare and low. In contrast, trout presented a distinct resistance profile, with four of the seven antibiotics (tetracycline, trimethoprim-sulfamethoxazole, ciprofloxacin and cefotaxime) showing moderate to extremely high resistance, while the remaining three antibiotics (ceftazidime, chloramphenicol, gentamicin) showed low resistance. The detail counts (frequency) of each cell in the heat map are presented in Annex I - Table 10.

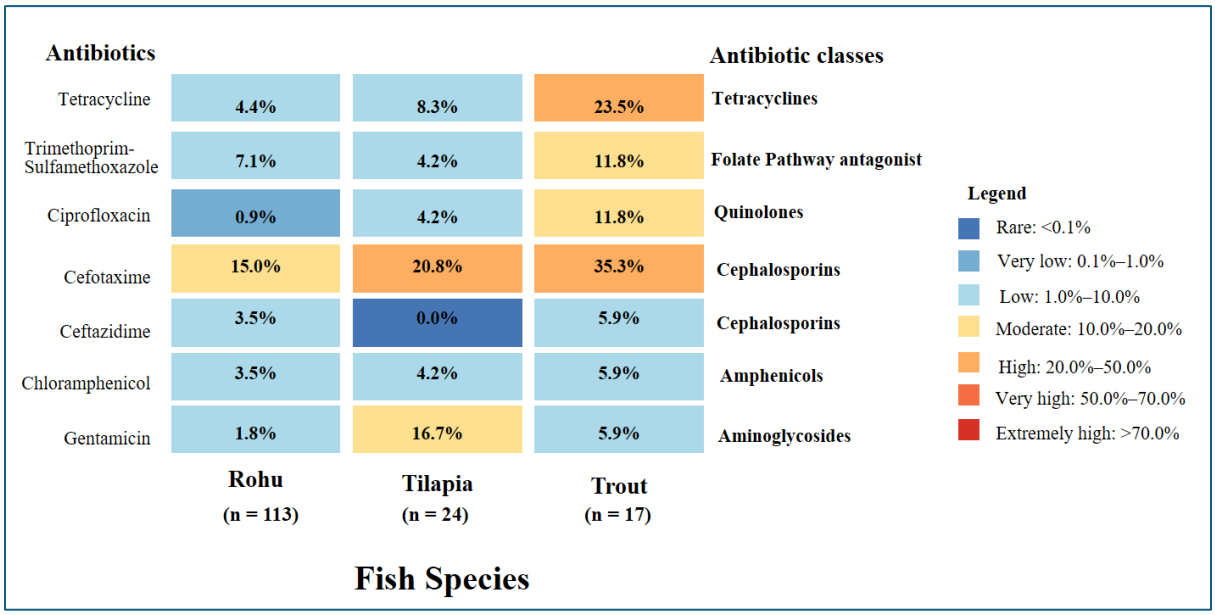

Figure 9: Heat map of resistance percentages in Aeromonas spp. isolated in rohu, tilapia and trout. The legend uses the EUSR's categorisation system.

3.2.2 AMR patterns based on WHO priority classification of bacterial isolates in fish

3.2.2.1 *E. coli* AMR in rohu

Figure 10 illustrates the AMR profile of 98 *E. coli* isolates recovered from healthy farmed rohu. Antibiotics are categorised according to the WHO classifications of HPCIA, CIA, HIA, and human-use only. Resistance varied substantially, ranging from 0.0% to 84.7%.

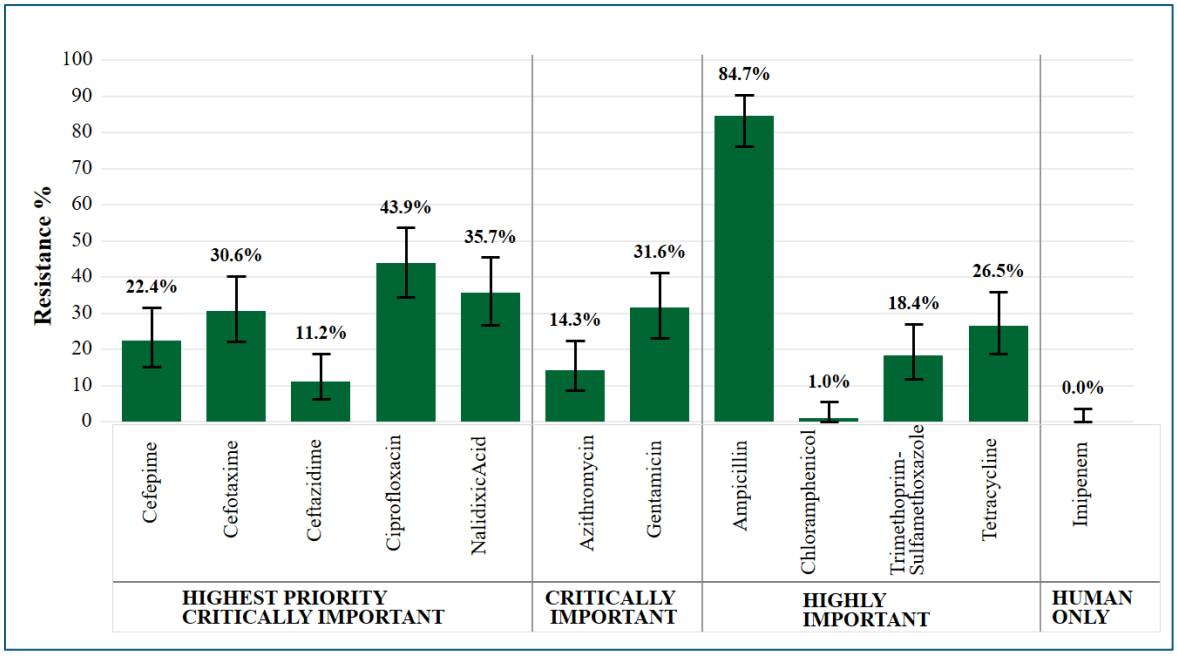

Figure 10: AST patterns of E. coli isolates in healthy rohu (n = 98) by WHO categorisation.

In the HPCIA category, cephalosporins and quinolones showed variable resistance: cefepime 22.4% (95% CI: 15.3–31.7%), cefotaxime 30.6% (95% CI: 22.4–40.3%), ceftazidime 11.2% (95% CI: 6.4–19.0%), ciprofloxacin 43.9% (95% CI: 34.5–53.7%), and nalidixic acid 35.7% (95% CI: 26.9–45.6%).

For CIA agents, gentamicin demonstrated 31.6% resistance (95% CI: 23.3–41.4%) and azithromycin 14.3% (95% CI: 8.7–22.6%).

Among HIA compounds, ampicillin showed the highest resistance at 84.7% (95% CI: 76.3–90.5%). Resistance to other HIA agents was chloramphenicol 1.0% (95% CI: 0.2–5.6%), trimethoprim-sulphamethoxazole 18.4% (95% CI: 11.9–27.2%), and tetracycline 26.5% (95% CI: 18.8–36.0%).

3.2.2.2 *E. coli* AMR in tilapia

Figure 11 presents the AMR profile of 35 *E. coli* isolates recovered from healthy farmed tilapia. Resistance varied in a similar pattern to rohu, ranging from 0.0% to 68.6%.

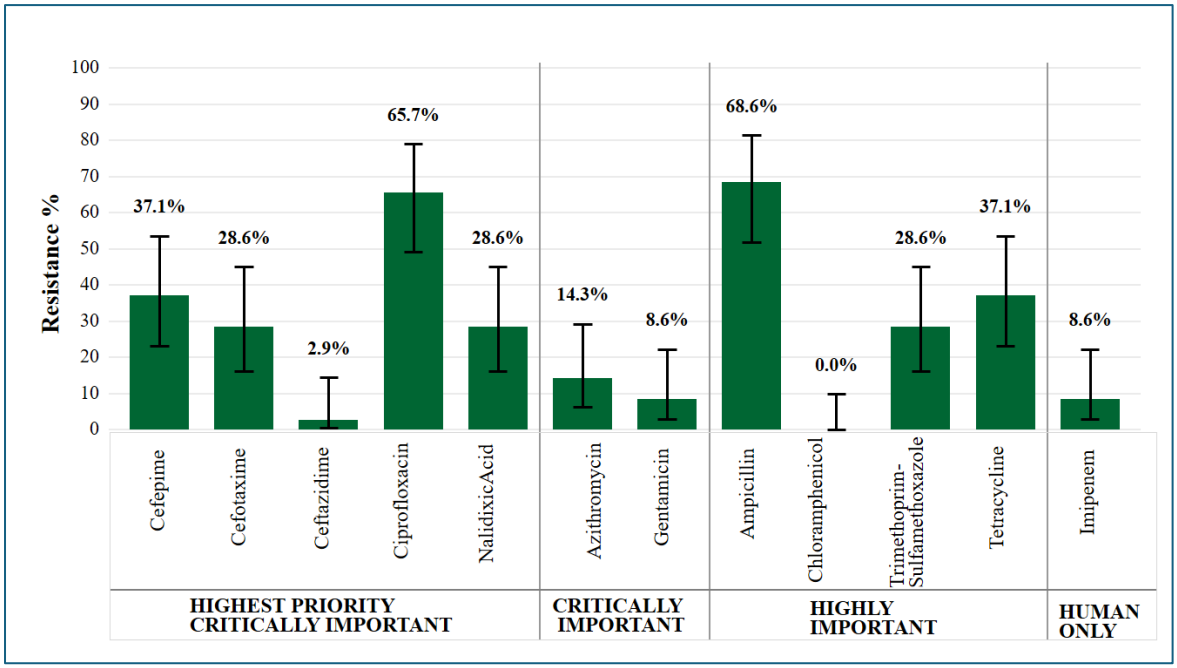

Figure 11: AST pattern of E. coli isolates in healthy tilapia (n = 35) by WHO categorisation.

Within the Human-use only category, imipenem exhibited 8.6% resistance (95% CI: 3 - 22.4%). *E. coli* isolated from tilapia were the only isolates that showed resistance to imipenem. *E. coli* from neither rohu nor trout showed resistance to imipenem.

In the HPCIA category, cephalosporins and quinolones showed variable resistance: cefepime 37.1% (95% CI: 23.2 - 53.7%), cefotaxime 28.6% (95% CI: 16.3 - 45.1%), ceftazidime 2.9% (95% CI: 0.5 - 14.5%), ciprofloxacin 65.7% (95% CI: 49.2 - 79.2%), and nalidixic acid 28.6% (95% CI: 16.3 - 45.1%).

For CIA agents, gentamicin demonstrated 8.6% resistance (95% CI: 3 - 22.4%) and azithromycin 14.3% (95% CI: 6.3 - 29.4%).

Among HIA compounds, ampicillin showed the highest resistance at 68.6% (95% CI: 52 - 81.4%). Resistance to other HIA agents was trimethoprim-sulfamethoxazole 28.6% (95% CI: 16.3 - 45.1%) and tetracycline 37.1% (95% CI: 23.2 - 53.7%). *E. coli* in tilapia was 100% susceptible to chloramphenicol.

3.2.2.3 *E. coli* AMR in trout

Figure 12 shows the AMR profile of 15 *E. coli* isolates recovered from healthy farmed trout. Resistance varied significantly, ranging from 0.0% to 73.3%. *E. coli* isolates from trout showed no resistance to six antibiotics.

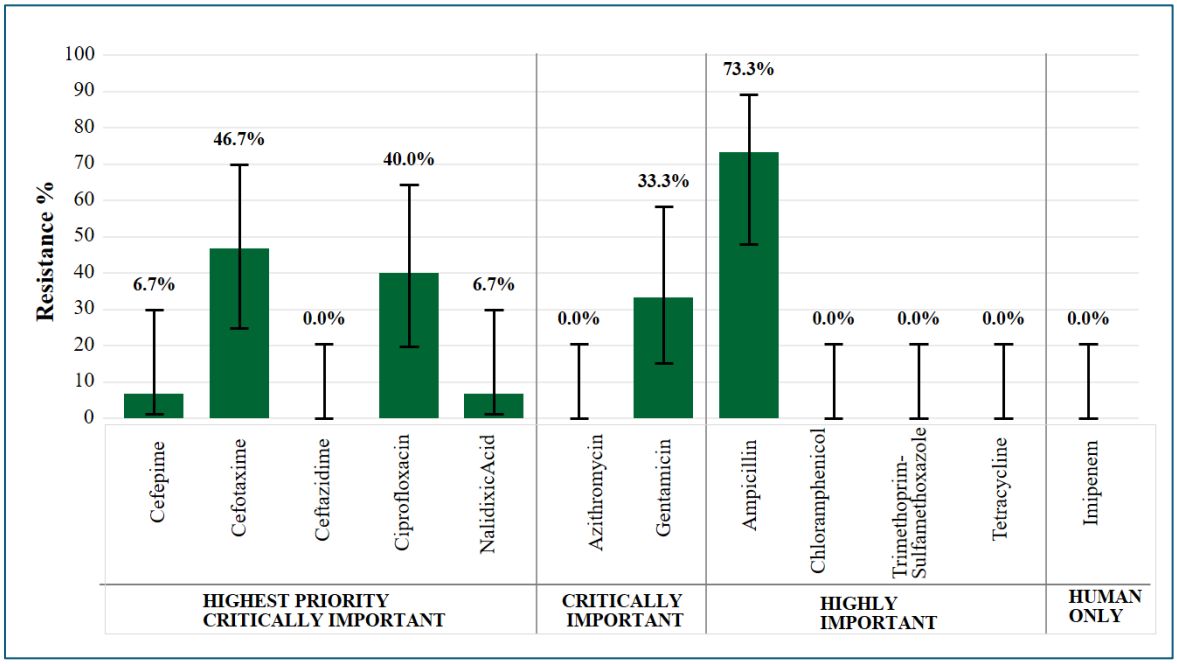

Figure 12: AST pattern of E. coli isolates in healthy trout (n = 15) by WHO categorisation.

In the HPCIA category, cephalosporins and quinolones showed differing resistance: cefepime 6.7% (95% CI: 1.2 - 29.8%), cefotaxime 46.7% (95% CI: 24.8 - 69.9%), ciprofloxacin 40% (95% CI: 19.8 - 64.3%), and nalidixic acid 6.7% (95% CI: 1.2 - 29.8%). *E. coli* in trout was 100% susceptible to ceftazidime.

For CIA agents, only gentamicin demonstrated 33.3% resistance (95% CI: 15.2 - 58.3%), whereas *E. coli* in trout was 100% susceptible to azithromycin.

Among HIA compounds, ampicillin showed the highest resistance at 73.3% (95% CI: 48 - 89.1%). *E. coli* in trout was 100% susceptible to chloramphenicol, trimethoprim-sulfamethoxazole, and tetracycline.

3.2.2.4 *Aeromonas* spp. AMR in rohu

Figure 13 presents the AMR profile of 113 *Aeromonas* spp. isolates recovered from healthy farmed rohu. Low levels of resistance were observed, ranging from 0.9% to 15.0%.

In the HPCIA category, the third generation cephalosporins and quinolones showed variable resistance: cefotaxime 15.0% (95% CI: 9.6 - 22.8%), ceftazidime 3.5% (95% CI: 1.4 - 8.7%) and ciprofloxacin 0.9% (95% CI: 0.2 - 4.8%).

For CIA, gentamicin demonstrated 1.8% resistance (95% CI: 0.5 - 6.2%).

Among HIA resistance percentage was chloramphenicol 3.5% (95% CI: 1.4 - 8.7%), trimethoprim-sulfamethoxazole 7.1% (95% CI: 3.6 - 13.4%), and tetracycline 4.4% (95% CI: 1.9 - 9.9%).

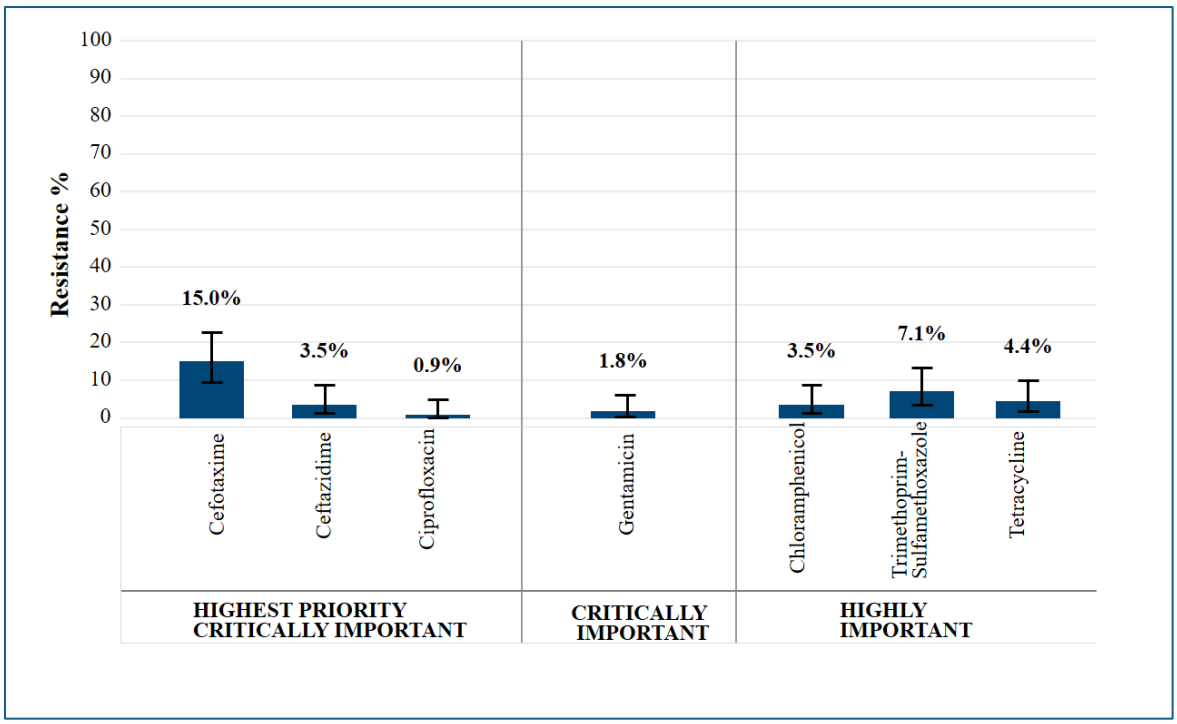

Figure 13: AST pattern of Aeromonas spp. isolates in healthy rohu (n = 113) by WHO categorisation.

3.2.2.5 *Aeromonas* spp. AMR in tilapia

Figure 14 illustrates the AMR profile of 24 *Aeromonas* spp. isolates recovered from healthy farmed tilapia. Varying levels of resistance were observed, ranging from 0.0% to 20.8%.

In the HPCIA category, the third generation cephalosporins and quinolones showed resistance: cefotaxime 20.8% (95% CI: 9.2 - 40.5%), ceftazidime 0% and ciprofloxacin 4.2% (95% CI: 0.7 - 20.2%).

For CIA, gentamicin demonstrated 16.7% resistance (95% CI: 6.7 - 35.9%).

HIA resistance percentage was chloramphenicol 4.2% (95% CI: 0.7 - 20.2%), trimethoprim-sulfamethoxazole 4.2% (95% CI: 0.7 - 20.2%), and tetracycline 8.3% (95% CI: 2.3 - 25.8%).

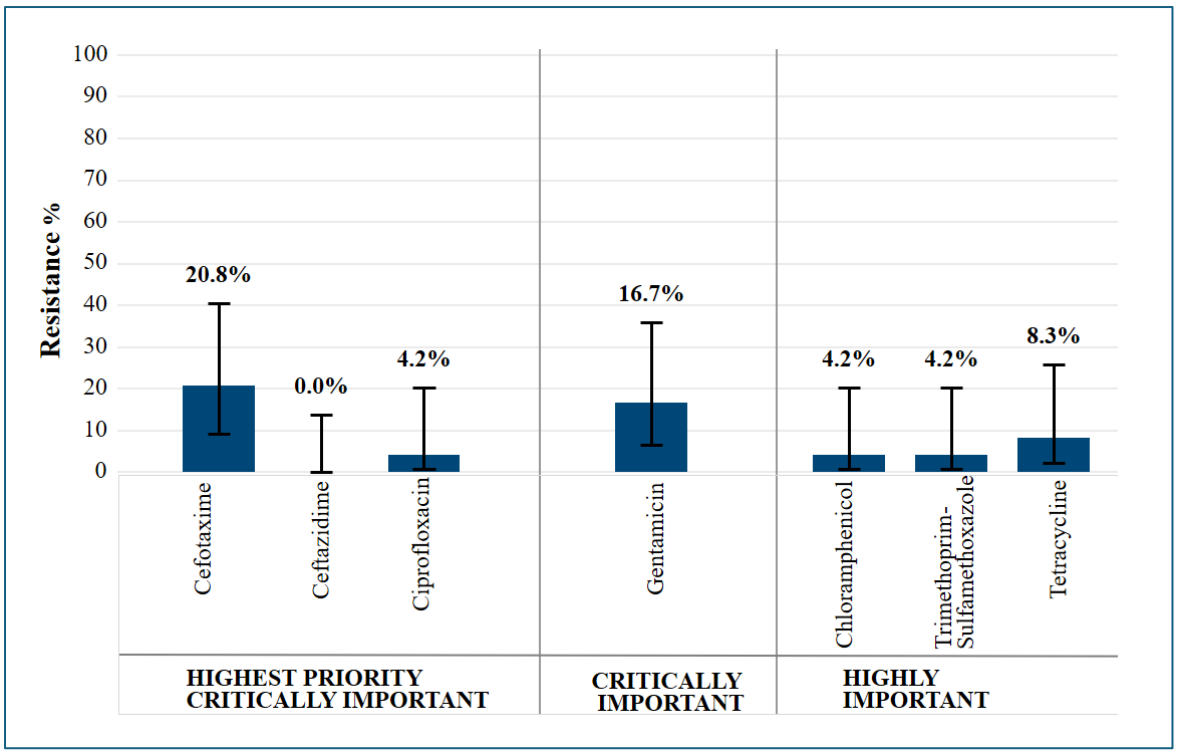

Figure 14: AST pattern of Aeromonas spp. isolates in healthy tilapia (n = 24) by WHO categorisation.

3.2.2.6 *Aeromonas spp.* AMR in trout

Figure 15 presents the AMR profile of 17 *Aeromonas* spp. isolates recovered from healthy farmed trout. Varying levels of resistance were observed, ranging from 5.9% to 35.3%.

In the HPCIA category, the third generation of cephalosporins and quinolones showed resistance: cefotaxime 35.3% (95% CI: 17.3 - 58.7%), ceftazidime 5.9% (95% CI: 1 – 27%) and ciprofloxacin 11.8% (95% CI: 3.3 - 34.3%).

For CIA, gentamicin demonstrated 5.9% resistance (95% CI: 1 – 27%).

Among HIA resistance percentage was chloramphenicol 5.9% (95% CI: 1 – 27%), trimethoprim-sulfamethoxazole 11.8% (95% CI: 3.3 - 34.3%), and tetracycline 23.5% (95% CI: 9.6 - 47.3%).

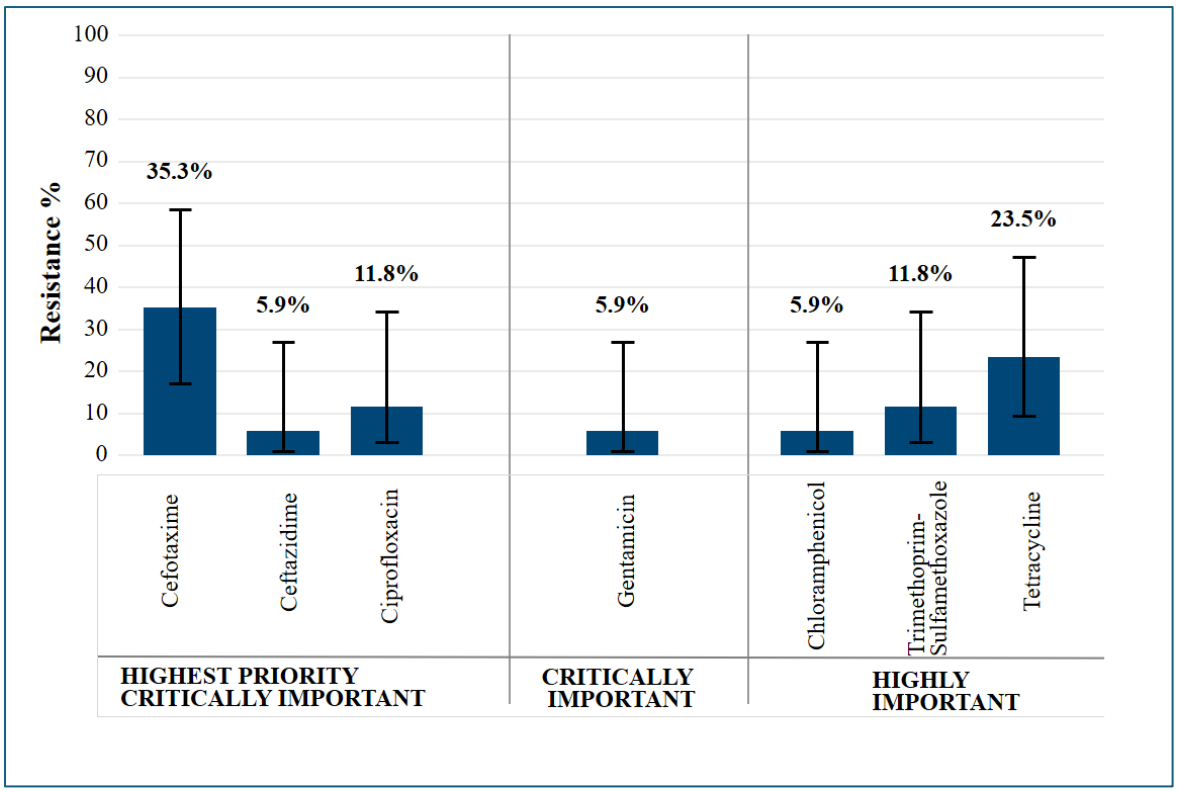

Figure 15: AST pattern of Aeromonas spp. isolates in healthy trout (n = 17) by WHO categorisation.

3.2.3 Multi-class resistance profile of bacterial isolates in fish

3.2.3.1 E. coli

The Figure 16 illustrates the multi-class resistance profile in *E. coli* isolates in rohu (n = 98), tilapia (n = 35) and trout (n = 15). Isolates exhibiting resistance to one or more antibiotics in three or more antibiotic classes were defined as multidrug-resistant (MDR)[29]. In rohu, MDR was observed in 38.8% of the total isolates. The remaining 61.2% were non-MDR (Annex I, Table 13). Within the non-MDR group, 3.1% of isolates were susceptible to all antibiotics in the AST panel. Resistance to a single antibiotic class was observed in 30.6% of isolates, predominantly to aminopenicillin. Resistance to two classes was identified in 27.6% of isolates, most commonly to a combination of aminopenicillin and quinolones (Annex I - Table 14).

Among the MDR *E. coli* isolates in rohu, resistance to three classes was found in 14.3%, with cephalosporin-aminopenicillin-quinolone resistance being the most frequent profile. Resistance to four classes occurred in 7.1% of isolates, primarily to cephalosporins, aminopenicillin, quinolones, and tetracyclines. Resistance to five different antibiotic class combinations was seen in 4.1% of isolates. Resistance to six classes was observed in 1.0% of isolates, while 12.2% exhibited resistance to all seven of the nine classes tested: aminoglycosides, cephalosporins, macrolides, aminopenicillin, quinolones, sulfonamides, and tetracyclines (Annexe I - Table 14).

Isolates from tilapia exhibiting MDR comprised of 48.6% of the total. The remaining 51.4% were non-MDR (Annex I - Table 13). Within the non-MDR group, 2.9% of isolates were fully susceptible to all agents on the AST panel. Resistance to a single antibiotic class was observed in 8.6% of isolates, predominantly to aminopenicillin. Resistance to two classes was identified in 40.0% of isolates, most commonly to a combination of aminopenicillin and quinolones. These patterns were same as *E. coli* isolated from rohu (Annexe I - Table 14).

Among the MDR *E. coli* isolates in tilapia, resistance to three classes was found in 22.9%, with cephalosporin-aminopenicillin-quinolone resistance being the most frequent profile. Resistance to four classes occurred in 11.4% of isolates. Resistance to five different antibiotic class combinations was seen in 5.7% of isolates. Resistance to six classes was observed in 5.7% of isolates, while 2.9% exhibited resistance to all seven of the nine classes tested: aminoglycosides, cephalosporins, macrolides, aminopenicillin, quinolones, sulfonamides, and tetracyclines (Annexe I - Table 14).

Isolates from trout exhibiting MDR comprised of 33.3% of the total. The remaining 66.7% were non-MDR. Within the non-MDR group, 6.7% of isolates were fully susceptible to all agents on the AST panel. Resistance to a single antibiotic class was observed in 33.3% of isolates. Resistance to two classes was identified in 26.7% of isolates.

Among the MDR *E. coli* isolates in trout, resistance to three classes was found in 13.3%, with cephalosporin-aminopenicillin-quinolone resistance being the most frequent profile. Resistance to four classes occurred in 20.0% of isolates, primarily to cephalosporins, aminopenicillin, quinolones, and tetracyclines.

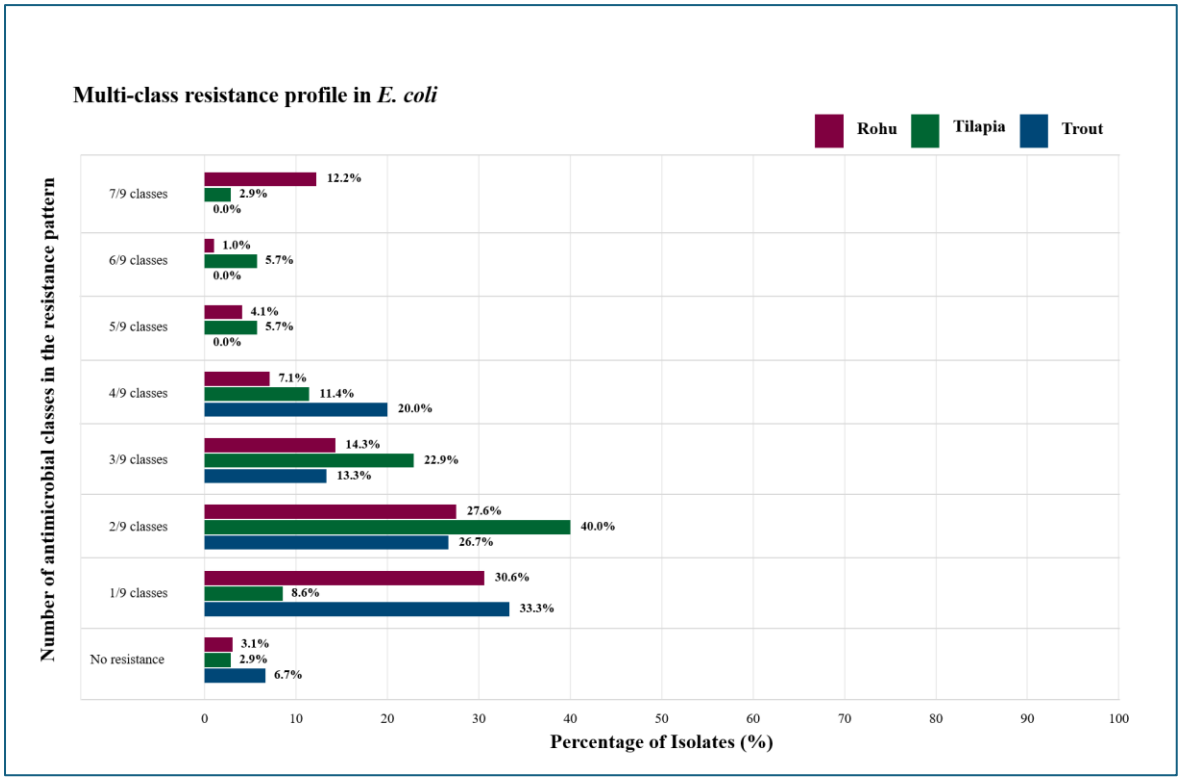

Figure 16: Multi-class resistance profile in E. coli for rohu (n = 98), tilapia (n = 35) and trout (n = 15) . Resistance was seen in as many as seven antibiotic classes.

3.2.3.2 *Aeromonas* spp.

The Figure 17 illustrates the multi-class resistance profile in *Aeromonas* isolates in rohu (n = 113), tilapia (n = 24) and trout (n = 17). Isolates from rohu exhibiting resistance to three or more antibiotic classes were defined as MDR, comprising 1.8% of the total. The remaining 98.2% were non-MDR (Annex I - Table 13). Within the non-MDR group, 76.1% of isolates were fully susceptible to all agents on the AST panel. Resistance to a single antibiotic class was observed in 19.5% of isolates, predominantly to cephalosporins. Resistance to two classes was identified in 2.7% of isolates, most commonly to a combination of cephalosporins and sulfonamides (Annex I - Table 15). MDR in *Aeromonas* spp. in all three fish species was much lower than the MDR observed in *E. coli* isolates.

Among the MDR *Aeromonas* spp. isolates in rohu, resistance to four classes occurred in 0.9% of isolates, resistance to five different antibiotic class combinations was seen in 0.9% of isolates (Annexe I - Table 15).

Isolates from tilapia exhibiting MDR against *Aeromonas* spp. comprised of 8.3% of the total. The remaining 91.7% were non-MDR (Table 13). Within the non-MDR group, 75.0% of isolates were fully susceptible to all agents on the AST panel. Resistance to a single antibiotic class was observed in 8.3% of isolates. Resistance to two classes was identified in 8.3% of isolates (Annexe I - Table 15).

Among the MDR *Aeromonas* spp. isolates in tilapia, resistance to three classes was found in 4.2%. Resistance to five different antibiotic class combinations was seen in 4.2% of isolates (Annexe I - Table 15).

Isolates from trout exhibiting MDR against *Aeromonas* spp. comprised of 5.9% of the total. The remaining 94.1% were non-MDR (Annexe I - Table 13). Within the non-MDR group, 52.9% of isolates were fully susceptible to all agents on the AST panel. Resistance to a single antibiotic class was observed in 23.5% of isolates, with predominant cephalosporin resistance. Resistance to two classes was identified in 17.6% of isolates (Annexe I - Table 15).

Among the MDR *Aeromonas* spp. isolates in trout, resistance to all six classes was found in 5.9% of isolates: aminoglycosides, amphenicols, cephalosporins, quinolones, sulfonamides and tetracyclines (Annex I - Table 15).

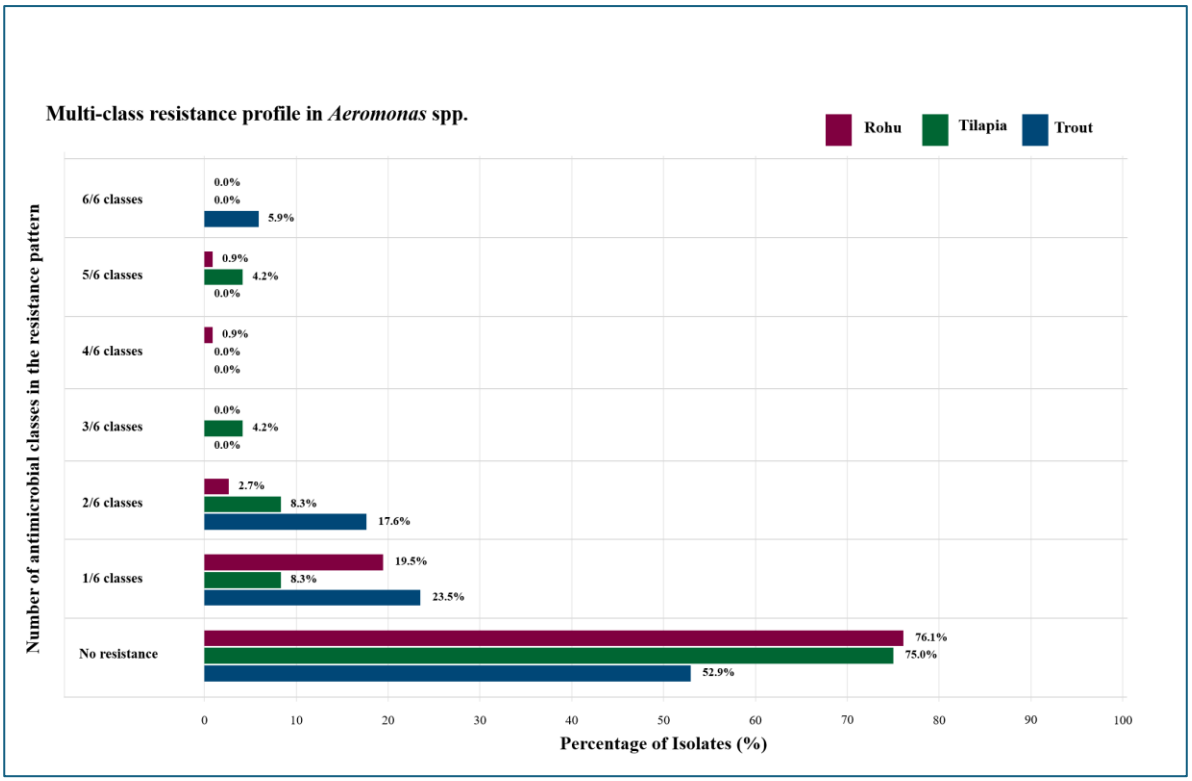

Figure 17: Multi-class resistance profile in Aeromonas spp. for rohu (n = 113), tilapia (n = 24) and trout (n = 17). Resistance was seen in as many as six antibiotic classes.

4. Discussion

This study investigated the prevalence of *E. coli* and *Aeromonas* spp. and their associated AMR profiles in healthy, farmed rohu, tilapia, and trout in Pakistan. The findings reveal a significant reservoir of resistant bacteria within the aquaculture system, with distinct patterns between bacterial species. The isolation percentages seen in the pilot study for *E. coli* are lower than the published literature[30–33] but that for *Aeromonas* was much higher[34–36].

With respect to MDR in *E. coli*, the high prevalence particularly to antibiotics of critical importance for human medicine, raises substantial public health concerns. These results are best interpreted through a One Health lens, recognizing the interconnectedness of human, animal, and environmental health[37,38]. Aquaculture sits at the intersection of these domains: agricultural practices influence environmental contamination, which impacts animal health and human health through the food chain and environmental exposure. It is critical to understand that AMR of *E. coli* represents the aquatic environment, whereas *Aeromonas* is an important member of fish gut microbiome that can cause diseases[33,39–42].

4.1 *Escherichia coli*

4.1.1 Isolation percentage and environmental context

The isolation of *E. coli* from the intestines of healthy fish (rohu 40.2%, tilapia 46.7%, trout 33.3%) is a clear indicator of faecal[43,44] contamination of the pond water. This is not an isolated phenomenon but a common issue in regions with intensive agriculture and human settlement. Research on surface waters in Southeast Asian cities has consistently demonstrated widespread faecal contamination, with *E. coli* serving as a key indicator bacterium of water pollution. The similar isolation rates across all three fish species, despite different aquatic ecologies, suggest a systemic issue where aquaculture ponds are influenced by contaminated water sources, such as rivers receiving untreated sewage or agricultural runoff[45]. This environmental reservoir is critical, as it continuously exposes fish and their gut microbiomes to bacteria from human and animal waste.

4.1.2 AMR phenotypes and linkage to regional drivers

The AMR profiles of *E. coli* are concerning. Across rohu and tilapia isolates, high levels of resistance were observed to commonly used antimicrobials in animals such as ampicillin, ciprofloxacin, and trimethoprim-sulfamethoxazole. Particularly noteworthy is the resistance to third-generation cephalosporins (e.g., cefotaxime) and the emergence of imipenem resistance in tilapia isolates (8.6%). These findings represent a significant escalation, as these antimicrobials are classified by WHO's HPCIAs and, in the case of carbapenems, reserved exclusively for human-use.

Table 8: Key E. coli AMR findings and regional context.

Antibiotic Class	Example	Significant Findings in Study (rohu/tilapia)	Context from Regional Research
Penicillins	Ampicillin	84.7% / 68.6% resistance	Consistently high resistance in clinical & environmental isolates in SEA at 48–55.2% [46].
Quinolones	Ciprofloxacin	43.9% / 65.7% resistance	High environmental concentrations in SEA wastewater select for resistance at 62·5% [45].
3rd-Gen Cephalosporins	Cefotaxime	30.6% / 28.6% resistance	Linked to dissemination of ESBL genes (e.g., *bla*CTX-M) in SEA water environments [44,47].

Antibiotic Class	Example	Significant Findings in Study (rohu/tilapia)	Context from Regional Research
Carbapenems	Imipenem	0% / 8.6% resistance	Signals advanced resistance; wastewater is a suspected hotspot for selection [45].
Multidrug Resistance (MDR)	≥3 classes	38.8% / 48.6% of isolates	MDR *E. coli* clones (e.g., ST131, ST410) are established in human and environmental cycles in SEA[48,49].

These patterns strongly mirror the AMR crisis in human medicine within the region. Clinical studies from SEA and beyond report similarly high AMR percentages in *E. coli* causing human infections (e.g., urinary tract infections) to ampicillin, ciprofloxacin, and trimethoprim-sulfamethoxazole[46]. This is not a coincidence but evidence of shared resistance genes and bacterial strains circulating among humans, animals, and the environment. The high MDR rates (up to 48.6%) are particularly concerning. Globally successful, high-risk MDR clones like sequence type (ST) 131 are prevalent in Southeast Asia, often carrying genes for extended-spectrum beta-lactamase (ESBL) production[48]. Another clone, ST410, has also been identified in humans and the environment in the region, carrying a unique set of resistance genes[49].

The current study did not identify the *E. coli* clones in fish, that limits further interpretations of the current results, though they clearly signify the intricate web of connection between human behaviour that directly impacts the environment and animals alike.

4.2 *Aeromonas* spp.

4.2.1 Isolation and Ecological Niche

Aeromonas spp. were isolated at a higher frequency than *E. coli* in rohu (46.3%) and trout (37.8%), reflecting their natural role as ubiquitous water-borne bacteria common in aquatic environments. Their comparatively lower prevalence in tilapia (32%) may reflect species-specific or pond management differences. Unlike *E. coli*, which is an enteric bacterium indicative of recent faecal pollution of water engulfed by the fish, *Aeromonas* is an autochthonous member of the aquatic microbiome. Its presence was expected, but its resistance profile is what warrants attention as an indicator of environmental AMR pressure.

It is noteworthy that all sentinel sites involved in the pilot study reported no therapeutic use of antimicrobials in their fish stocks, as no disease outbreaks had been observed. They however, reported the application of poultry litter and livestock manure to fertilise ponds prior to stocking. This practice is a common a cost-effective means of promoting algal growth to provide natural feed for the fish[50,51]. The implications of this fertilisation method for AMR selection requires further consideration, since antibiotics are widely used in poultry production[52].

4.2.2 AMR Phenotypes and Environmental Selection Pressure

The overall AMR levels in *Aeromonas* spp. were much lower than in *E. coli*, particularly for rohu and tilapia isolates. *Aeromonas* from trout exhibited a notably high level of resistance profile, with higher resistance to tetracycline, trimethoprim-sulfamethoxazole, ciprofloxacin, and cefotaxime. This suggests that the cold-water trout farming environment may be subject to different or more intense antimicrobial selection pressures, even though the water sources are much more pristine in the north of Pakistan than south where rohu and tilapia are harvested.

The detection of MDR *Aeromonas* [up to 8.3% in tilapia (2/22)], including one isolate resistant to five of the six classes tested, demonstrates that environmental bacteria in these systems are slowly

acquiring and accumulating resistance determinants. The results were even more intriguing in trout, where only one of 16 isolates showed resistance to all six classes of antibiotics, bring MDR to 5.9%. *Aeromonas* spp. can act as a reservoir for resistance genes in the environment, with the potential for horizontal gene transfer to other bacteria, including pathogens[44].

4.3 Public Health Implications and the Imperative for a One Health Approach

The public health implications of this study are profound. Healthy fish destined for human consumption are contaminated with bacteria resistant to first line and last-resort antibiotics.
This poses a dual risk:

1. Direct transmission: MDR bacteria can colonize the human gut through improper handling or undercooking of fish, potentially leading to hard-to-treat infections.
2. Gene Transfer Reservoir: The fish gut and the aquaculture environment serve as mixing vessels where resistant bacteria from human, animal, and environmental sources can exchange resistance genes, amplifying the and spread of AMR.

The findings reflect the seepage of AMR from human and terrestrial animal sources into aquatic ecosystems, where they are recovered from fish. The high levels of resistance to HPCIA's like ciprofloxacin and cephalosporins found in this study in both *E. coli* and *Aeromonas* spp., are especially worrying given that antibiotic residues from these very classes are frequently detected in SEA waterways as a result of human contamination at concentrations high enough to promote resistance[6,44,45,53]. Molecular confirmation of the resistance genes is critical in future studies in Pakistan to provide evidence for the said pathway.

4.4 Limitations and Way forward

Addressing this complex threat requires a One Health approach, mobilizing coordinated action across human health, animal health, and environmental sectors. The data presented in this report provides valuable insights into AMR patterns in Pakistan's inland farmed fish sectors. However, it is essential to exercise caution when interpreting these findings, as the data is based on only nine months of surveillance, with only eight sentinel sites and healthy fish. This is a key methodological limitation, where exclusive reliance on phenotypic AST was done without molecular confirmation of resistance mechanisms (e.g., ESBL, carbapenemase genes). This limits the ability to track specific genetic determinants of resistance. This fundamental limitation underscores why these preliminary data are not suitable for regulatory or trade decisions. Therefore, it is crucial to expand the AMR surveillance and collect more comprehensive longitudinal data to inform effective policy change that is supported by molecular confirmation of resistance mechanisms. Instead, the following is recommended:

1. Continue surveillance for an extended period to capture seasonal variations and trends in a larger variety of fish species and bacteria.
2. Increase the sample size to ensure representative data.
3. Include molecular confirmation of resistance (e.g., ESBL, carbapenemase genes) to identify source of AMR.
4. Expand the scope of surveillance to include additional bacterial species, antibiotic classes, and sectors (e.g., environment).
5. Foster collaborative efforts among stakeholders to develop and implement effective AMR mitigation strategies.
6. AMU studies in aquaculture is crucial to determine the possible causes and sources of AMR in aquaculture.

Aquaculture systems, influenced by broader environmental contamination, act as a reservoir and potential amplifier for MDR bacteria. By adopting a cautious and rigorous approach, we can ensure that the findings of this surveillance initiative are reliable, accurate, and actionable, ultimately contributing to the development of evidence-based policies and strategies to combat AMR in the aquaculture sector in Pakistan.

5. Conclusion

In conclusion, this pilot study provides initial evidence that inland aquaculture in Pakistan reflects a wider environmental AMR crisis, with fish serving as sentinels for contamination by MDR bacteria originating from human and terrestrial animal sources. The findings highlight the sector as a potential reservoir and exposure pathway within the broader One Health landscape. The distinct patterns in *Aeromonas* further highlight the role of the environment in AMR selection. These findings are not isolated but are part of a larger regional AMR crisis fuelled by environmental contamination. Mitigating this threat is impossible through isolated sectoral interventions. A proactive, collaborative, and well-resourced One Health approach is the only viable strategy to safeguard the efficacy of antibiotics for future generations.

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Annex I

Table 9: Antibiotic susceptibility of E. coli isolates from fish species, categorized by WHO priority classification, antibiotic class, and resistance phenotypes categorized as resistant (R), intermediate (I), and susceptible (S). [CIA = Critically Important antibiotics, HIA= Highly important antibiotics, HPCIA= Highest priority critically important antibiotics].

Fish Species	WHO Category	Class	Antibiotic	Resistant (R)	Intermediate (I)	Susceptible (S)	Total Isolates
Rohu	CIA	Aminoglycoside	Gentamicin	19	12	67	98
Rohu	CIA	Macrolide	Azithromycin	14	0	84	98
Rohu	HIA	Aminopenicillin	Ampicillin	77	6	15	98
Rohu	HIA	Amphenicol	Chloramphenicol	1	0	97	98
Rohu	HIA	Sulfonamide + trimethoprim	Trimethoprim-Sulphamethoxazole	16	2	80	98
Rohu	HIA	Tetracycline	Tetracycline	21	5	72	98
Rohu	HPCIA	3rd gen Cephalosporin	Cefotaxime	15	15	68	98
Rohu	HPCIA	3rd gen Cephalosporin	Ceftazidime	11	0	87	98
Rohu	HPCIA	4th gen Cephalosporin	Cefepime	15	7	76	98
Rohu	HPCIA	Quinolone	Ciprofloxacin	21	22	55	98
Rohu	HPCIA	Quinolone	NalidixicAcid	20	15	63	98
Rohu	Human_Only	Carbapenem	Imipenem	0	0	98	98
Tilapia	CIA	Aminoglycoside	Gentamicin	3	0	32	35
Tilapia	CIA	Macrolide	Azithromycin	4	1	30	35
Tilapia	HIA	Aminopenicillin	Ampicillin	19	5	11	35
Tilapia	HIA	Amphenicol	Chloramphenicol	0	0	35	35
Tilapia	HIA	Sulfonamide + trimethoprim	Trimethoprim-Sulphamethoxazole	9	1	25	35
Tilapia	HIA	Tetracycline	Tetracycline	11	2	22	35
Tilapia	HPCIA	3rd gen Cephalosporin	Cefotaxime	2	8	25	35
Tilapia	HPCIA	3rd gen Cephalosporin	Ceftazidime	1	0	34	35
Tilapia	HPCIA	4th gen Cephalosporin	Cefepime	10	3	22	35
Tilapia	HPCIA	Quinolone	Ciprofloxacin	10	13	12	35
Tilapia	HPCIA	Quinolone	NalidixicAcid	4	6	25	35
Tilapia	Human_Only	Carbapenem	Imipenem	3	0	32	35
Trout	CIA	Aminoglycoside	Gentamicin	2	3	10	15
Trout	CIA	Macrolide	Azithromycin	0	0	15	15
Trout	HIA	Aminopenicillin	Ampicillin	11	0	4	15
Trout	HIA	Amphenicol	Chloramphenicol	0	0	15	15
Trout	HIA	Sulfonamide + trimethoprim	Trimethoprim-Sulphamethoxazole	0	0	15	15
Trout	HIA	Tetracycline	Tetracycline	0	0	15	15
Trout	HPCIA	3rd gen Cephalosporin	Cefotaxime	0	7	8	15
Trout	HPCIA	3rd gen Cephalosporin	Ceftazidime	0	0	15	15
Trout	HPCIA	4th gen Cephalosporin	Cefepime	0	1	14	15
Trout	HPCIA	Quinolone	Ciprofloxacin	1	5	9	15
Trout	HPCIA	Quinolone	NalidixicAcid	0	1	14	15

Trout	Human_Only	Carbapenem	Imipenem	0	0	15	15

Table 10: Antibiotic susceptibility of Aeromonas spp. isolates from fish species, categorized by WHO priority classification, antibiotic class, and resistance phenotypes categorized as resistant (R), intermediate (I), and susceptible (S). [CIA = Critically Important antibiotics, HIA= Highly important antibiotics, HPCIA= Highest priority critically important antibiotics]

Fish Species	WHO Category	Class	Antibiotic	Resistant (R)	Intermediate (I)	Susceptible (S)	Total Isolates
Rohu	CIA	Aminoglycoside	Gentamicin	2	0	111	113
Rohu	HIA	Amphenicol	Chloramphenicol	1	3	109	113
Rohu	HIA	Sulfonamide + trimethoprim	Trimethoprim-Sulphamethoxazole	7	1	105	113
Rohu	HIA	Tetracycline	Tetracycline	3	2	108	113
Rohu	HPCIA	3rd gen Cephalosporin	Cefotaxime	4	13	96	113
Rohu	HPCIA	3rd gen Cephalosporin	Ceftazidime	4	0	109	113
Rohu	HPCIA	Quinolone	Ciprofloxacin	1	0	112	113
Tilapia	CIA	Aminoglycoside	Gentamicin	4	0	20	24
Tilapia	HIA	Amphenicol	Chloramphenicol	0	1	23	24
Tilapia	HIA	Sulfonamide + trimethoprim	Trimethoprim-Sulphamethoxazole	1	0	23	24
Tilapia	HIA	Tetracycline	Tetracycline	1	1	22	24
Tilapia	HPCIA	3rd gen Cephalosporin	Cefotaxime	1	4	19	24
Tilapia	HPCIA	3rd gen Cephalosporin	Ceftazidime	0	0	24	24
Tilapia	HPCIA	Quinolone	Ciprofloxacin	0	1	23	24
Trout	CIA	Aminoglycoside	Gentamicin	0	1	16	17
Trout	HIA	Amphenicol	Chloramphenicol	0	1	16	17
Trout	HIA	Sulfonamide + trimethoprim	Trimethoprim-Sulphamethoxazole	2	0	15	17
Trout	HIA	Tetracycline	Tetracycline	2	2	13	17
Trout	HPCIA	3rd gen Cephalosporin	Cefotaxime	1	5	11	17
Trout	HPCIA	3rd gen Cephalosporin	Ceftazidime	1	0	16	17
Trout	HPCIA	Quinolone	Ciprofloxacin	1	1	15	17

Table 11: Antibiotic resistance profile for E. coli in fish species showing resistance rates (R%) with 95% confidence intervals. R= Resistant, I= Intermediate.

Fish Species	WHO Category	Antibiotic	R+I	Total isolates	R%	95% CI
Rohu	CIA	Gentamicin	31	98	31.6	23.3 - 41.4
Rohu	CIA	Azithromycin	14	98	14.3	8.7 - 22.6
Rohu	HIA	Ampicillin	83	98	84.7	76.3 - 90.5
Rohu	HIA	Chloramphenicol	1	98	1	0.2 - 5.6
Rohu	HIA	Trimethoprim-Sulphamethoxazole	18	98	18.4	11.9 - 27.2
Rohu	HIA	Tetracycline	26	98	26.5	18.8 - 36
Rohu	HPCIA	Cefotaxime	30	98	30.6	22.4 - 40.3
Rohu	HPCIA	Ceftazidime	11	98	11.2	6.4 - 19
Rohu	HPCIA	Cefepime	22	98	22.4	15.3 - 31.7

Rohu	HPCIA	Ciprofloxacin	43	98	43.9	34.5 - 53.7
Rohu	HPCIA	NalidixicAcid	35	98	35.7	26.9 - 45.6
Rohu	Human Only	Imipenem	0	98	0	0 - 3.8
Tilapia	CIA	Gentamicin	3	35	8.6	3 - 22.4
Tilapia	CIA	Azithromycin	5	35	14.3	6.3 - 29.4
Tilapia	HIA	Ampicillin	24	35	68.6	52 - 81.4
Tilapia	HIA	Chloramphenicol	0	35	0	0 - 9.9
Tilapia	HIA	Trimethoprim-Sulphamethoxazole	10	35	28.6	16.3 - 45.1
Tilapia	HIA	Tetracycline	13	35	37.1	23.2 - 53.7
Tilapia	HPCIA	Cefotaxime	10	35	28.6	16.3 - 45.1
Tilapia	HPCIA	Ceftazidime	1	35	2.9	0.5 - 14.5
Tilapia	HPCIA	Cefepime	13	35	37.1	23.2 - 53.7
Tilapia	HPCIA	Ciprofloxacin	23	35	65.7	49.2 - 79.2
Tilapia	HPCIA	NalidixicAcid	10	35	28.6	16.3 - 45.1
Tilapia	Human Only	Imipenem	3	35	8.6	3 - 22.4
Trout	CIA	Gentamicin	5	15	33.3	15.2 - 58.3
Trout	CIA	Azithromycin	0	15	0	0 - 20.4
Trout	HIA	Ampicillin	11	15	73.3	48 - 89.1
Trout	HIA	Chloramphenicol	0	15	0	0 - 20.4
Trout	HIA	Trimethoprim-Sulphamethoxazole	0	15	0	0 - 20.4
Trout	HIA	Tetracycline	0	15	0	0 - 20.4
Trout	HPCIA	Cefotaxime	7	15	46.7	24.8 - 69.9
Trout	HPCIA	Ceftazidime	0	15	0	0 - 20.4
Trout	HPCIA	Cefepime	1	15	6.7	1.2 - 29.8
Trout	HPCIA	Ciprofloxacin	6	15	40	19.8 - 64.3
Trout	HPCIA	NalidixicAcid	1	15	6.7	1.2 - 29.8
Trout	Human Only	Imipenem	0	15	0	0 - 20.4

Table 12: Antibiotic resistance profile for Aeromonas spp. in fish species showing resistance rates (R%) with 95% confidence intervals. R= Resistant, I= Intermediate

Fish Species	WHO Category	Antibiotic	R+I	Total Isolates	R%	95% CI
Rohu	CIA	Gentamicin	2	113	1.8	0.5 - 6.2
Rohu	HIA	Chloramphenicol	4	113	3.5	1.4 - 8.7
Rohu	HIA	Trimethoprim-Sulphamethoxazole	8	113	7.1	3.6 - 13.4
Rohu	HIA	Tetracycline	5	113	4.4	1.9 - 9.9
Rohu	HPCIA	Cefotaxime	17	113	15.0	9.6 - 22.8
Rohu	HPCIA	Ceftazidime	4	113	3.5	1.4 - 8.7
Rohu	HPCIA	Ciprofloxacin	1	113	0.9	0.2 - 4.8
Tilapia	CIA	Gentamicin	4	24	16.7	6.7 - 35.9
Tilapia	HIA	Chloramphenicol	1	24	4.2	0.7 - 20.2
Tilapia	HIA	Trimethoprim-Sulphamethoxazole	1	24	4.2	0.7 - 20.2
Tilapia	HIA	Tetracycline	2	24	8.3	2.3 - 25.8
Tilapia	HPCIA	Cefotaxime	5	24	20.8	9.2 - 40.5
Tilapia	HPCIA	Ceftazidime	0	24	0.0	0 - 13.8

Tilapia	HPCIA	Ciprofloxacin	1	24	4.2	0.7 - 20.2
Trout	CIA	Gentamicin	1	17	5.9	1 - 27
Trout	HIA	Chloramphenicol	1	17	5.9	1 - 27
Trout	HIA	Trimethoprim-Sulphamethoxazole	2	17	11.8	3.3 - 34.3
Trout	HIA	Tetracycline	4	17	23.5	9.6 - 47.3
Trout	HPCIA	Cefotaxime	6	17	35.3	17.3 - 58.7
Trout	HPCIA	Ceftazidime	1	17	5.9	1 - 27
Trout	HPCIA	Ciprofloxacin	2	17	11.8	3.3 - 34.3

Table 13: Multi-drug resistance (MDR) and Non-MDR resistance percentage in E. coli and Aeromonas in fish species.

Bacterium	Fish Species	Total Samples	MDR Count	Non-MDR Count	MDR %	Non-MDR %
E. coli	Rohu	98	38	60	38.8	61.2
E. coli	Tilapia	35	17	18	48.6	51.4
E. coli	Trout	15	5	10	33.3	66.7
Aeromonas	Rohu	113	2	111	1.8	98.2
Aeromonas	Tilapia	24	2	22	8.3	91.7
Aeromonas	Trout	17	1	16	5.9	94.1

Table 14: Distribution of MDR patterns among E. coli isolates from rohu, tilapia and trout detailing the specific combination of antibiotic classes, the number of classes resisted, and the frequency of each pattern.

Fish specie	Combination of antibiotic-resistant classes	# of antibiotic classes resistant	# of isolates with the specific resistance pattern	Total isolates
Rohu	None	0	3	98
Rohu	Aminoglycosides	1	3	98
Rohu	Penicillins	1	21	98
Rohu	Quinolones	1	5	98
Rohu	Tetracyclines	1	1	98
Rohu	Aminoglycosides + Penicillins	2	6	98
Rohu	Aminoglycosides + Quinolones	2	1	98
Rohu	Cephalosporins + Penicillins	2	5	98
Rohu	Cephalosporins + Quinolones	2	2	98
Rohu	Penicillins + Quinolones	2	11	98
Rohu	Penicillins + Tetracyclines	2	2	98
Rohu	Aminoglycosides + Penicillins + Quinolones	3	5	98
Rohu	Cephalosporins + Penicillins + Quinolones	3	6	98
Rohu	Cephalosporins + Penicillins + Tetracyclines	3	1	98
Rohu	Penicillins + Quinolones + Tetracyclines	3	2	98
Rohu	Aminoglycosides + Cephalosporins + Penicillins + Quinolones	4	1	98
Rohu	Cephalosporins + Penicillins + Quinolones + Sulfonamides	4	1	98
Rohu	Cephalosporins + Penicillins + Quinolones + Tetracyclines	4	4	98
Rohu	Penicillins + Quinolones + Sulfonamides + Tetracyclines	4	1	98
Rohu	Aminoglycosides + Amphenicols + Penicillins + Quinolones + Tetracyclines	5	1	98

Rohu	Aminoglycosides + Cephalosporins + Penicillins + Sulfonamides + Tetracyclines	5	1	98
Rohu	Cephalosporins + Macrolides + Penicillins + Quinolones + Sulfonamides	5	1	98
Rohu	Cephalosporins + Penicillins + Quinolones + Sulfonamides + Tetracyclines	5	1	98
Rohu	Aminoglycosides + Cephalosporins + Macrolides + Penicillins + Quinolones + Sulfonamides	6	1	98
Rohu	Aminoglycosides + Cephalosporins + Macrolides + Penicillins + Quinolones + Sulfonamides + Tetracyclines	7	12	98
Tilapia	None	0	1	35
Tilapia	Cephalosporins	1	1	35
Tilapia	Penicillins	1	2	35
Tilapia	Cephalosporins + Penicillins	2	2	35
Tilapia	Cephalosporins + Sulfonamides	2	1	35
Tilapia	Macrolides + Penicillins	2	1	35
Tilapia	Penicillins + Quinolones	2	6	35
Tilapia	Quinolones + Sulfonamides	2	1	35
Tilapia	Quinolones + Tetracyclines	2	2	35
Tilapia	Sulfonamides + Tetracyclines	2	1	35
Tilapia	Aminoglycosides + Quinolones + Tetracyclines	3	1	35
Tilapia	Cephalosporins + Macrolides + Sulfonamides	3	1	35
Tilapia	Cephalosporins + Penicillins + Quinolones	3	2	35
Tilapia	Cephalosporins + Quinolones + Sulfonamides	3	1	35
Tilapia	Cephalosporins + Quinolones + Tetracyclines	3	1	35
Tilapia	Penicillins + Quinolones + Tetracyclines	3	2	35
Tilapia	Cephalosporins + Penicillins + Quinolones + Sulfonamides	4	2	35
Tilapia	Cephalosporins + Penicillins + Quinolones + Tetracyclines	4	2	35
Tilapia	Carbapenems + Cephalosporins + Macrolides + Penicillins + Quinolones	5	1	35
Tilapia	Cephalosporins + Penicillins + Quinolones + Sulfonamides + Tetracyclines	5	1	35
Tilapia	Aminoglycosides + Carbapenems + Penicillins + Quinolones + Sulfonamides + Tetracyclines	6	1	35
Tilapia	Carbapenems + Cephalosporins + Macrolides + Penicillins + Quinolones + Tetracyclines	6	1	35
Tilapia	Aminoglycosides + Cephalosporins + Macrolides + Penicillins + Quinolones + Sulfonamides + Tetracyclines	7	1	35
Trout	none	0 (fully susceptible)	1	15
Trout	Aminoglycosides	1	1	15
Trout	Cephalosporins	1	1	15
Trout	Penicillins	1	2	15
Trout	Quinolones	1	1	15
Trout	Cephalosporins + Penicillins	2	2	15
Trout	Penicillins + Quinolones	2	2	15
Trout	Aminoglycosides + Cephalosporins + Penicillins	3 (MDR)	1	15
Trout	Cephalosporins + Penicillins + Quinolones	3 (MDR)	1	15
Trout	Aminoglycosides + Cephalosporins + Penicillins + Quinolones	4 (MDR)	3	15

Table 15: Distribution of MDR patterns among Aeromonas isolates from Rohu, tilapia, trout detailing the specific combination of antibiotic classes, the number of classes resisted, and the frequency of each pattern.

Fish specie	Combination of antibiotic-resistant classes	# of antibiotic classes resistant	# of isolates with the specific resistance pattern	Total isolates
Rohu	None	0 (fully susceptible)	86	113
Rohu	Amphenicols	1	2	113
Rohu	Cephalosporins	1	13	113
Rohu	Sulfonamides	1	3	113
Rohu	Tetracyclines	1	4	113
Rohu	Amphenicols + Sulfonamides	2	1	113
Rohu	Cephalosporins + Sulfonamides	2	2	113
Rohu	Aminoglycosides + Amphenicols + Cephalosporins + Sulfonamides	4 (MDR)	1	113
Rohu	Aminoglycosides + Cephalosporins + Quinolones + Sulfonamides + Tetracyclines	5 (MDR)	1	113
Tilapia	None	0 (fully susceptible)	18	24
Tilapia	Aminoglycosides	1	1	24
Tilapia	Cephalosporins	1	1	24
Tilapia	Aminoglycosides + Cephalosporins	2	2	24
Tilapia	Cephalosporins + Quinolones + Tetracyclines	3 (MDR)	1	24
Tilapia	Aminoglycosides + Amphenicols + Cephalosporins + Sulfonamides + Tetracyclines	5 (MDR)	1	24
Trout	None	0	9	17
Trout	Tetracyclines	1	1	17
Trout	Cephalosporins	1	3	17
Trout	Cephalosporins + Sulfonamides	2	1	17
Trout	Quinolones + Tetracyclines	2	1	17
Trout	Cephalosporins + Tetracyclines	2	1	17
Trout	Aminoglycosides + Amphenicols + Cephalosporins + Quinolones + Sulfonamides + Tetracyclines	6	1	17

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