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RESEARCH ARTICLE

Prevalence and Antibiotic Resistance Profiles of Quinolone-Resistant Bacteria from Freshwater Fish Farms in Southern Punjab, Pakistan

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ABSTRACT

Quinolone resistance in bacteria poses a global health threat, and aquaculture environments may act as reservoirs for resistant strains and their genes. We investigated the prevalence, antimicrobial resistance profiles, and key genetic determinants of quinolone resistance by screening for associated resistance genes among 101 bacterial isolates from water and sediment at freshwater fish-farming sites in four districts of Southern Punjab, Pakistan (Bahawalpur, Lodhran, Multan, Muzaffargarh). Isolates were tested for susceptibility to enrofloxacin, ciprofloxacin, and norfloxacin via disk diffusion. Phenotypic prevalence and genetic associations were analyzed using Chi-square tests. Resistant isolates underwent PCR screening for plasmid-mediated quinolone resistance genes (*qnrA*, *qnrB*, *qnrS*) and mutations in the quinolone resistance–determining regions (QRDR) of *gyrA* and *gyrB*. Selected resistant isolates were identified by 16S rRNA sequencing. Phenotypic resistance was 30% (95% CI: 21-39) for norfloxacin, 27% (95% CI: 18-36) for enrofloxacin, and 18% (95% CI: 10-26) for ciprofloxacin. District-wise significant variations were observed for enrofloxacin (P=0.01) and ciprofloxacin (P<0.001) resistance. Among the three-plasmid mediated quinolone resistance (PMQR) genes (*qnrA*, *qnrB*, *qnrS*) screened, a significant association was found between the presence of these genes and phenotypic resistance to enrofloxacin (P=0.03), ciprofloxacin (P=0.05), and norfloxacin (P=0.01). Among all three PMQR genes, *qnrS* was the most frequently detected gene (37-40%), followed by *qnrA* (27-33%). District-level resistance to enrofloxacin was highest in Multan water (53%) and Bahawalpur sediment (44%), but low (0–10%) in Muzaffargarh and Lodhran. Ciprofloxacin resistance was predominately seen in Bahawalpur sediment (50%) and water from Multan (29%) and Muzaffargarh (25%), with none in Lodhran. Norfloxacin resistance peaked in Muzaffargarh water (50%), followed by Multan water (41%) and sediment (40%), with overall greater susceptibility in water isolates. Isolates had high multi-antibiotic resistance indices (~0.5), indicated anthropogenic pollution. Species identified included *Pseudomonas aeruginosa* and *Achromobacter xylosoxidans*. These findings highlight the dissemination of PMQR genes in multidrug-resistant opportunistic pathogens. We recommend strengthened antimicrobial stewardship, improved biosecurity, and routine AMR surveillance in aquaculture to protect animal, public, and environmental health.

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INTRODUCTION

Fish farming is a sector that has experienced very rapid growth and is fundamental as a part of the solution to global food security, with innovations in fish farming leading to higher production rates (Dubey et al., 2022; Milijasevic et al., 2024). However, intensified farming has led to frequent bacterial disease outbreaks and thus a frequent use of antibiotics, primarily quinolones, to control infections (Das et al., 2018; John et al., 2023). Too many antibiotics used without thinking have caused antimicrobial resistance (AMR) and multidrug-resistant (MDR) bacteria to grow in

water, putting biodiversity and public health at risk (Pepi and Focardi, 2021; Hossain et al., 2022). The public health impact of AMR is particularly severe in Pakistan, where it was the third leading cause of death in 2019, with an estimated 59,200 deaths directly attributable to bacterial AMR (Safdar et al., 2025). National surveillance data confirms this pressure on the human health sector, with *Escherichia coli* being the most frequently isolated pathogen and showing significant resistance to multiple drug classes (Zhang et al., 2024). Pakistan's aquaculture industry, especially in Punjab, has grown thanks to tilapia farming, carp polyculture, and the country's many freshwater resources, such as the Indus River (Abro et al., 2020; Rossignoli et al., 2023). However, overfishing, pollution, and the overuse of antibiotics have damaged habitats and made bacteria more resistant (Manzoor et al., 2023). Previous research in Pakistan has established that local aquaculture environments serve as reservoirs for antimicrobial resistance genes (ARGs). A landmark study identified multiple ARGs, including *blaTEM*, in integrated fish farms in Pakistan even without a recorded history of direct antibiotic use, suggesting that inputs like agricultural waste contribute to a background pool of resistance (Shah et al., 2012). More recently, studies have confirmed the presence of multidrug-resistant pathogens like *Aeromonas hydrophila* carrying resistance and virulence genes in fish from Punjab's rivers and farms (Mahmood et al., 2024a; Mahmood et al., 2024b).

Quinolones are broad-spectrum antibiotics used in human and veterinary medicine for treating bacterial infections (Gram-positive and Gram-negative). The World Health Organization (WHO) recommends reducing their use in livestock due to the rapid development of quinolone-resistant strains (WHO, 2017). These antimicrobials inhibit bacterial DNA replication by targeting DNA *gyrA*se and topoisomerase IV, leading to chromosomal fragmentation and bacterial cell death (Aldred et al., 2014). Bacteria acquire resistance to quinolones through mutations in quinolone-resistance determining regions (QRDRs) of genes encoding these DNA topoisomerases (*gyrA, gyrB, parC, parE*), which diminish drug-binding affinity (Correia et al., 2017). An increasing rate of quinolone resistance has been reported in bacteria isolated from various sources, including food-producing animals, food products, and environmental samples (Padilla and Amatorio, 2017; Paraoan et al., 2017; Vital et al., 2017; Torio and Padilla, 2018).

The plasmid-borne *qnr* genes, a key mediator of quinolone resistance, was first identified in *Klebsiella pneumoniae* in 1998 (Martinez-Martinez et al., 1998) and subsequently detected in numerous other bacterial species (Mammeri et al., 2005; Karah et al., 2010). To date, three primary families of *qnr* genes (*qnrA*, *qnrB*, and *qnrS*) have been characterized, exhibiting nucleotide identity differences of 40% or greater (Correia et al., 2017). Initial reports of *qnrA* and *qnrS* in Scandinavia originated from Denmark, with *qnrS* subsequently detected in Norway (Karah et al., 2010: Shah et al., 2012).

The presence of plasmid-mediated quinolone resistance (PMQR) genes is extremely widespread in both clinical and environmental settings around the world. PMQR genes have been reported in around 25% of *Escherichia coli* isolates, regardless of their source of isolation (highest prevalence: 49% found in retail turkey meat in the Czech Republic) (Wang et al., 2024). Of 160 PMQR-positive *E. coli* isolates, 30% were from aquatic environment samples in China, with a greater occurrence (28%) in hospital-affected water as well as in aquaculture-affected water, where 37% of the isolates were PMQR-positive (Röderova et al., 2017). PMQR genes have been found in *Klebsiella pneumoniae* and *E. coli* isolates from human infections, poultry, and water sources that are contaminated in South-East Asia (Pakistan and Bangladesh). A 2024 study of wastewater in Pakistan found PMQR genes like *qnrS1* and *aac(6')-Ib-cr* in over 77% of tested environmental isolates (Sattar et al., 2024). Furthermore, extreme pharmaceutical pollution, including quinolones, has been documented in major rivers like the Ravi, creating intense selective pressure in regional aquatic ecosystems (Wilkinson et al., 2022). Previous studies highlight the distribution of quinolone-resistant bacteria as a serious health threat and a danger to public health and food safety (Rahman et al., 2017; Mahmud et al., 2018).

The increasing use of antibiotics in aquaculture to combat disease outbreaks, coupled with the close interaction between terrestrial and aquatic bacteria, poses a significant threat of AMR dissemination in fish farms (Marti et al., 2014; Cabello et al., 2016; Schar et al., 2020). Despite this evidence of ARGs in Pakistani fish farms and a high prevalence of PMQR genes in local wastewater, a clear knowledge gap remains. There is a lack of systematic surveillance quantifying the prevalence of specific PMQR genes (*qnrA, qnrB, qnrS*) and their link to phenotypic quinolone resistance at the farm level within aquaculture itself. Recognizing this, Pakistan has recently developed a National AMR Surveillance Strategy for Aquaculture, prioritizing the generation of such baseline data to inform policy (Qiu et al., 2024). While previous studies have documented antibiotic contamination and AMR in aquatic environments, there is a gap in knowledge regarding the prevalence of PMQR genes and chromosomal mutations conferring quinolone resistance specifically in fish farming sites within Southern Punjab region (Cantas et al., 2013; Woo et al., 2022). Our study directly addresses this critical gap. Therefore, this study aimed to investigate water and sediment samples from aquatic environments in Southern Punjab (Bahawalpur, Lodhran, Multan, Muzaffargarh) to determine the presence of PMQR genes (*qnrA*, *qnrB*, *qnrS*) and mutations in QRDRs of chromosomal genes (*gyrA*, *gyrB*), as well as resistance to enrofloxacin, ciprofloxacin, and norfloxacin.

MATERIALS AND METHODS

Study Area and Sample Collection: This study was conducted in four major freshwater fish farming districts of Southern Punjab, Pakistan Bahawalpur, Lodhran, Multan, and Muzaffargarh purposively selected due to their high concentration of intensive aquaculture activities and their representation of diverse agricultural landscapes within the region. Environmental samples were collected comprising both pond water and sediment periodically throughout November 2023 and October 2024 to encompass all major seasons (summer, autumn, and winter). While a larger number of environmental samples were initially processed, the final dataset consists of 101 viable bacterial isolates that

were successfully cultivated, purified, and characterized for this study. For water samples, 10ml was aseptically collected from three distinct layers (upper surface, middle, and bottom) of each pond and pooled into a sterile 30ml plastic bottle. Sediment samples were collected from the pond bottom using a sterile pipette, and approximately 1g of sediment was immediately mixed with 0.9ml of sterile saline solution (0.9% NaCl) to maintain viability. All samples were carefully labeled with the collection site, date, and location. Samples were then kept on ice and taken to the postgraduate laboratory at the Department of Zoology, Cholistan University of Veterinary and Animal Sciences (CUVAS), Bahawalpur, for microbiological testing.

Bacterial Isolation and Cultivation: Environmental samples were subjected to 10-fold serial dilutions in sterile saline solution. Aliquots (30µL) of appropriate dilutions were spread-plated onto Luria Bertani (LB) agar (NEOGEN, UK), a non-selective medium, to facilitate the growth of diverse bacterial species. LB agar was prepared by autoclaving 20g agar powder in 50mL distilled water at 121°C for 15 minutes and poured into 30mm diameter disposable Petri plates under aseptic conditions in Biosafety Cabinet (ESCO Class II, Singapore). Plates were incubated at 37°C for 24 hours. Distinct colonies, differentiated by morphological characteristics (size, shape, color), were selected and repeatedly streaked onto fresh LB agar plates to obtain pure cultures. Purified isolates were preserved at -20°C in LB broth supplemented with 20% Glycerol (Addgene, USA) for subsequent analyses.

Phenotypic Screening for Quinolone Resistance: All purified bacterial isolates were initially screened for phenotypic resistance to quinolones. Antibiotic susceptibility testing (AST) was performed using the Kirby-Bauer disk diffusion method on Mueller-Hinton Agar (MHA) (Oxoid, UK) plates, following Clinical and Laboratory Standards Institute (CLSI) guidelines where applicable, with adaptations for environmental isolates. Briefly, pure isolates were cultured in Luria Bertani broth (LB) at 37°C for 18–24 hours to achieve a turbidity equivalent to a 0.5 McFarland standard (approximately 1.5 × 10^8CFU/mL). Bacterial suspensions were then uniformly swabbed onto MHA plates.

Commercial antibiotic-impregnated disks (Oxoid, UK) for three quinolones Enrofloxacin (5µg) (Bioanalyse, Turkey), Ciprofloxacin (5µg) (Biomaxima, Poland) and Norfloxacin (10µg) (Bioanalyse, Turkey) were applied. Plates were incubated at 37°C for 18–24 hours. Inhibition zone diameters (IZDs) were measured using digital calipers. Interpretation of susceptibility (susceptible and resistant) was based on established epidemiological cut-off values or guidelines appropriate for environmental bacteria proposed by Kronvall (2003) and Smith et al. (2009). Isolates exhibiting resistance to at least one of the tested quinolones were prioritized for further molecular characterization. The Multiple Antibiotic Resistance (MAR) index was calculated as the number of antibiotics to which an isolate was resistant divided by the total number of antibiotics tested (Krumperman, 1983; Kumarasamy et al., 2010).

Molecular Detection of Quinolone Resistance Genes: Genomic DNA was extracted from pure cultures of quinolone-resistant isolates using the WizPrep™ gDNA Mini Kit (Wizbio Solutions, South Korea) according to the manufacturer's instructions. The extracted DNA was quantified and its purity assessed using a NanoDrop Spectrophotometer (PG Instruments, UK).

PCR amplification was performed to detect common plasmid-mediated quinolone resistance (PMQR) genes (*qnrA*, *qnrB*, *qnrS*) and mutations in quinolone resistance-determining regions (QRDRs) of chromosomal genes (*gyrA*, *gyrB*). Primers and PCR conditions including specific annealing temperatures were adapted from previously published studies (Table 1). Each 25µL PCR reaction mixture contained 12µL of 2X Master Mix (EmeraldAmp GT PCR Master Mix, Takara Bio), 1µL of each forward and reverse primer (Oligo Factory, USA) (10pmol/µL), 5 µL of template DNA (approx. 50-100 ng), and nuclease-free cell culture water (BIOWEST, France). Nuclease-free water was used as the negative control in all PCR assays to monitor for contamination. PCR was performed on a C1000 Touch Thermal Cycler (Bio-Rad, USA) with the following general cycling conditions: initial denaturation at 95°C for 5 minutes; 35 cycles of denaturation at 95°C for 30 seconds, annealing at the gene-specific temperature for 1 minute, and extension at 72°C for 45 seconds; followed by a final extension at 72°C for 10 minutes.

Amplified PCR products were resolved by Electrophoresis (Bio-Rad, USA) on 1.5% agarose gel (Thermo Scientific, USA) stained with CYBR™ Green Nucleic Acid Gel Stain (Invitrogen, USA) in 1X TAE buffer (Thermo Scientific, USA). A 100bp DNA ladder (Takara Bio, Japan) was used as a molecular weight marker. Gels were visualized under a UV transilluminator using a gel documentation system (Bio-Rad, USA). For confirmation, representative PCR products of expected sizes for quinolone resistance genes were excised from the gel, purified using the WizPrep™ Gel/PCR Purification Mini Kit (Wizbio Solutions, South Korea), and sent for Sanger sequencing to Macrogen Inc. (South Korea). The resulting DNA sequences for these resistance genes were carefully analyzed. The identity of each gene was confirmed by Sanger sequencing of the purified amplicons, which served as the ultimate positive control by verifying that the amplified product was the intended target gene.

Species Identification of Selected Isolates: Selected isolates exhibiting phenotypic quinolone resistance and carrying confirmed quinolone resistance genes were subjected to species identification through 16S rRNA gene sequencing. The 16S rRNA gene was amplified using universal primers (27F: AGAGTTTGATCCTGGCTCAG and 1492R: GGTTACCTTGTTACGACTT). PCR products were purified and sent for Sanger sequencing to Macrogen Inc. (South Korea). The obtained 16S rRNA gene sequences were compared against the NCBI 16S ribosomal RNA sequences database using Basic Local Alignment Search Tool (BLAST) for species identification. The 16S rRNA gene sequences obtained in this study were submitted to the National Center for Biotechnology Information (NCBI) GenBank database and assigned accession numbers PV255000-PV255017.

Phylogenetic Analysis: Resulting DNA sequences from 16S rRNA gene sequencing were analyzed using the BLASTn at the NCBI GenBank database to identify the closest match species. We have clarified that sequencing was bidirectional, and a consensus sequence was assembled after visual inspection of chromatograms. We have also specified the thresholds used for species identification: ≥99% sequence identity with a query coverage of >98%. Multiple sequence alignment of the obtained sequences, along with selected reference sequences from GenBank, was performed using ClustalW. Phylogenetic analysis was conducted using the neighbor-joining (NJ) method in Molecular Evolutionary Genetics Analysis (MEGA) software version 12.0.11 (Kumar et al., 2024). The robustness of the resulting phylogenetic tree topology was evaluated by bootstrap analysis with 1000 replicates.

Statistical Analysis: Data on phenotypic resistance prevalence, MAR indices, and gene detection frequencies were recorded in Microsoft Excel. Descriptive statistics (percentages, frequencies) were calculated. Chi-square tests or Fisher's exact tests were used, where appropriate, to compare the prevalence of quinolone resistance and resistance genes among different districts and sample sources (water and sediment), with a P-value<0.05 considered statistically significant.

RESULTS

Phenotypic Quinolone Resistance Prevalence: A total of 101 bacterial isolates were successfully recovered from the environmental samples and analyzed in this study. A notable level of resistance to quinolones was observed, with 27% (27/101) of isolates exhibiting resistance to enrofloxacin, 18% (18/101) to ciprofloxacin, and 30% (30/101) to norfloxacin (Table 2). The prevalence of resistance to each of these quinolones was statistically significant (P<0.001).

District-Wise and Source-Specific Distribution of Quinolone Resistance: The prevalence of phenotypic resistance to quinolones demonstrated considerable variation across the four sampled districts and between environmental sources (water and sediment), as detailed in Table 3. Resistance to enrofloxacin varied significantly across the districts ($P = 0.01$), with the highest prevalence observed in water samples from Multan (53%, 9/17) and sediment samples from Bahawalpur (44%, 7/16), while no enrofloxacin resistance was detected in any isolates from Muzaffargarh. Ciprofloxacin resistance also showed significant inter-district variation ($P<0.001$). Notably, Bahawalpur sediment samples exhibited the highest ciprofloxacin resistance at 50% (8/16), whereas Multan sediment samples showed a low prevalence of 5% (1/20), and no ciprofloxacin resistance was found in isolates from Lodhran. For norfloxacin, while overall inter-district variation was not statistically significant ($P = 0.39$), resistance levels were relatively high in Multan (41% in water, 7/17; 40% in sediment, 8/20) and Lodhran (43% in water, 3/7; 30% in sediment, 3/10), with Muzaffargarh water samples also showing 50% resistance (2/4) albeit from a small sample size.

Detection of Quinolone Resistance Genes in Phenotypically Resistant Isolates: Isolates exhibiting phenotypic resistance to enrofloxacin (N=27), ciprofloxacin (N=18), and norfloxacin (N=30) were further screened for the presence of common PMQR genes (*qnrA*, *qnrB*, *qnrS*) (Table 4). Fig. 1 shows gel electrophoresis results following PCR amplification of the *qnr* genes. Among the 27 enrofloxacin-resistant isolates, *qnrS* was the most frequently detected gene (37.0%, 10/27), followed by *qnrA* (33.3%, 9/27). The *gyrA* and *gyrB* genes were found in 11.1% (3/27) and 7.4% (2/27) of these isolates, respectively. Of the 18 ciprofloxacin-resistant isolates, *qnrS* was again the most prevalent gene (38.9%, 7/18). *qnrA* was detected in 33.3% (6/18) of isolates. In the 30 norfloxacin-resistant isolates, *qnrS* exhibited the highest frequency at 40.0% (12/30). This was followed by *qnrA* (26.7%, 8/30). The *qnrB* genes was detected less frequently, each in 6.7% (2/30) of norfloxacin-resistant isolates (Fig. 2). However, screening of chromosomal

Table 1: Primer Sequences, Product Size and Annealing Temperature for the Detection of Quinolone Resistance Genes by PCR

Target Gene	Primer Sequence (5' to 3')	Amplicon Size (bp)	Annealing Temp.	Reference
qnrA	F: TCAGCAAGAGGATTTCTCA R: GGCAGCACTATTACTCCCA	627	58°C	(Shah et al., 2012)
qnrB	F: GATCGTGAAAGCCAGAAAGG R: ACGATGCCTGGTAGTTGTCC	496	56°C	(Shah et al., 2012)
qnrS	F: ACGACATTCGTCAACTGCAA R: TAAATTGGCACCCTGTAGGC	417	56°C	(Shah et al., 2012)
gyrA	F: CGAGATGGACGCCAAGGAA R: GCGAATCCTCTTCCACCTCAAC	991	61°C	(Pang et al., 2018)
gyrB	F: ACCAAGACCAAACTGGGCAACA R: CGGAACAGCAGCGTCAACAA	540	60°C	(Altamia et al., 2014; Pang et al., 2018)

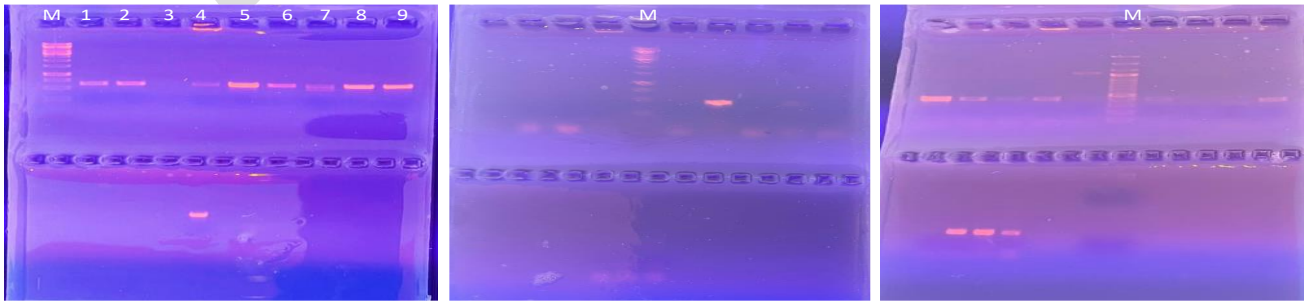

Fig. 1: Representative gel electrophoresis of PCR amplicons for quinolone resistance genes. (A) *qnrA* (627 bp); (B) *qnrB* (496 bp); (C) *qnrS* (417 bp). Lane M: 100 bp DNA ladder. Positive lanes show amplicons of the expected size from representative quinolone-resistant isolates.

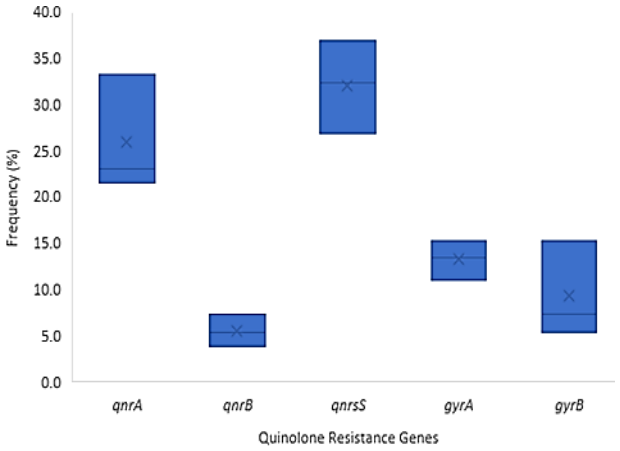

Fig. 2: Frequency of quinolone resistance genes.

Table 2: Overall Prevalence of Phenotypic Resistance to Selected Quinolones among Bacterial Isolates (n=101)

Antibiotic	Resistant (Nr)	Susceptible	PR (%)	95% CI	χ^2	*P* value
Enrofloxacin	27	74	27	18-36	19.96	<0.001
Ciprofloxacin	18	83	18	10-26	39.18	<0.001
Norfloxacin	30	71	30	21-39	14.98	<0.001

Nr = Number of resistant isolates; PR (%) = Prevalence of resistance; 95% CI = 95% Confidence Interval. χ^2 and P value derived from Chi-square test comparing observed vs. expected frequencies (Expected Resistant=49.44, Expected Susceptible=51.56).

Table 3: District-Wise and Source-Specific Prevalence of Phenotypic Resistance to Quinolones

Antibiotic Overall χ^2 (p-value)	District	Water (Nr/Nt, PR%)	Sediment (Nr/Nt, PR%)
Enrofloxacin 18.22 (*P*=0.01)	Bahawalpur	3/22 (14%)	7/16 (44%)
	Lodhran	0/7 (0%)	1/10 (10%)
	Multan	9/17 (53%)	7/20 (35%)
	Muzaffargarh	0/4 (0%)	0/5 (0%)
Ciprofloxacin 20.29 (*P*<0.001)	Bahawalpur	3/22 (14%)	8/16 (50%)
	Lodhran	0/7 (0%)	0/10 (0%)
	Multan	5/17 (29%)	1/20 (5%)
	Muzaffarghar	1/4 (25%)	0/5 (0%)
Norfloxacin 7.35 (*P*=0.39)	Bahawalpur	4/22 (18%)	2/16 (13%)
	Lodhran	3/7 (43%)	3/10 (30%)
	Multan	7/17 (41%)	8/20 (40%)
	Muzaffargarh	2/4 (50%)	1/5 (20%)

Nr = Number of resistant isolates; Nt = Total number of isolates tested from that source/district. PR (%) = Prevalence of resistance. P value derived from Chi-square test for overall variation in resistance prevalence across all districts and sources for each antibiotic.

Table 4: Association Between Phenotypic Resistance and Presence of *qnr* Genes

Phenotypic Resistance	N	Gene Detected	Positive (n)	% of Resistant	Total χ^2	*P* value
Enrofloxacin	27	*qnrA*	9	33.3		
		qnrB	2	7.4		
		qnrS	10	37.0	7.33	0.03
Ciprofloxacin	18	*qnrA*	6	33.3		
		qnrB	1	5.6		
		qnrS	7	38.9	5.98	0.05
Norfloxacin	30	*qnrA*	8	26.7		
		qnrB	2	6.7		
		qnrS	12	40.0	9.14	0.01

N = Total number of phenotypically resistant isolates screened for genes. n = Number of isolates positive for a specific gene. χ^2 and P value derived from Chi-square test for association between phenotypic resistance and the presence of any qnr gene.

QRDR of *gyrA*, *gyrB* did not show any mutation. The MAR index for different isolates ranged between 0.2 and 1, supporting the hypothesis that antimicrobial contamination in these environments originates from external sources, such as aquaculture practices and animal waste (Cow dung, poultry waste etc.) being used in the fish ponds. Chi-square analysis was performed to determine if a statistical association existed between the observed phenotypic resistance to a specific quinolone and the presence of any of the tested *qnr* genes (*qnrA, qnrB, qnrS*). A significant association was found for enrofloxacin, where the presence of a *qnr* gene was strongly linked to phenotypic resistance (χ^2 = 7.33, *P*=0.03). A similar significant association was observed for norfloxacin (χ^2 = 8.62, *P*=0.01). However, for ciprofloxacin, the association between phenotypic resistance and the presence of a *qnr* gene did not reach statistical significance (χ^2 = 5.40, *P*=0.07) (Table 4).

Species Identification of Quinolone-Resistant Isolates Harboring Resistance Genes: To characterize the bacterial species carrying quinolone resistance determinants, three representative isolates that were phenotypically resistant and positive for PMQR genes were successfully identified via 16S rRNA gene sequencing. The species-wise distribution of the detected quinolone resistance genes for these identified isolates was as follows: *Pseudomonas aeruginosa*: Two isolates were identified as *P. aeruginosa*. Both of these isolates (100%, 2/2) were positive for the *qnrA* gene. *Achromobacter xylosoxidans*: One isolate was identified as *A. xylosoxidans*. This isolate was screened for all PMQR genes but was found to be negative for them. The phylogenetic relationships of these isolates are shown in Fig. 3-5. Isolate H4_contig_1 (*P. aeruginosa*) and H5_contig_1 (*P. aeruginosa*) showed high sequence identities of 99.86% and 99.79% respectively, while H11_contig_1 (*A. xylosoxidans*) showed 99.93% identity to their respective type strains.

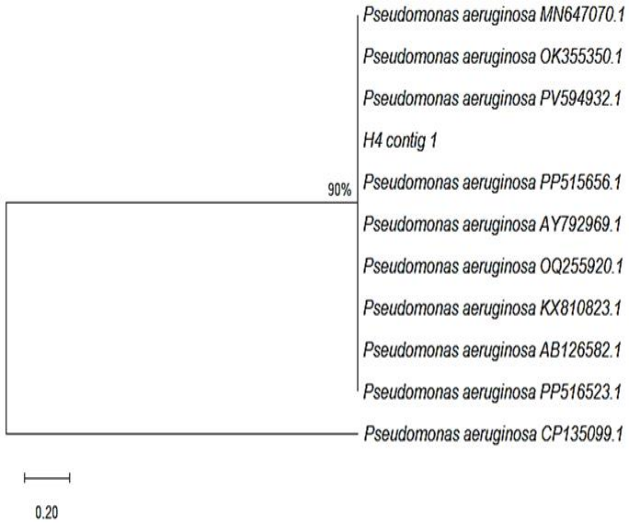

Fig. 3: Phylogenetic tree of 16S rRNA generated by using MEGA 12.0.11 adjusting the bootstrap values of 1000. H4 isolate (positive for *qnrA*) identified as *Pseudomonas aeruginosa*. Isolate H4_contig_1 clustered within a clade consisting entirely of *P. aeruginosa* sequences. It suggests that the *qnrA* resistance determinant may have been acquired through horizontal gene transfer from a common ancestor or related *P. aeruginosa* circulating within fish farming environment.

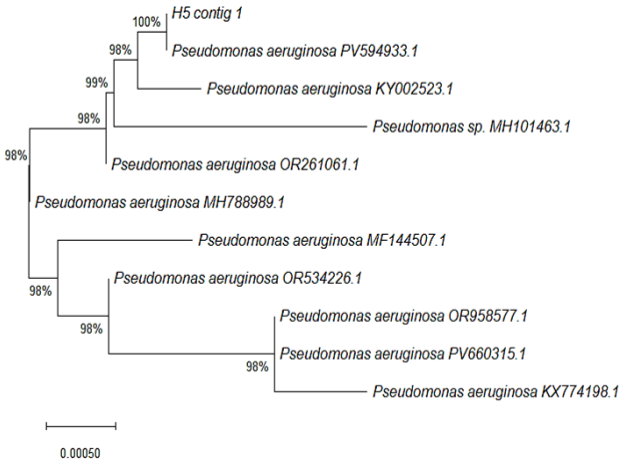

Fig. 4: Phylogenetic tree of 16S rRNA generated by using MEGA 12.0.11 adjusting the bootstrap values of 1000. H5 isolate (positive for *qnrA*) identified as *Pseudomonas aeruginosa*. Isolate H5_contig_1 formed a well-supported clade (100% bootstrap value) with *Pseudomonas aeruginosa* PV594933.1 and KY002523.1, branching from a slightly broader clade (98% bootstrap value) that also included *Pseudomonas* sp. MH101463.1 and *Pseudomonas aeruginosa* OR261061.1. The tree topology indicates a close evolutionary relationship between isolate H5_contig_1 and these diverse environmental or clinical *P. aeruginosa* strains. While this analysis confirms the identification of H5_contig_1 as *Pseudomonas aeruginosa*, the relatively low genetic distance between these strains suggests a recent divergence or active gene flow within the *P. aeruginosa* population, emphasizing the potential for the rapid dissemination of quinolone resistance determinants like *qnrA* among these closely related bacteria.

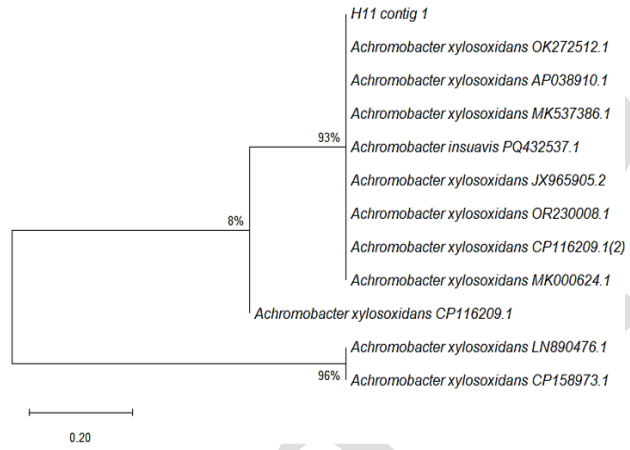

Fig. 5: Phylogenetic tree of 16S rRNA generated by using MEGA 12.0.11 adjusting the bootstrap values of 1000. H11 isolate (positive for *gyrB*) identified as *Achromobacter xylosoxidans*. The tree topology reveals that H11_contig_1 shares a close evolutionary relationship with several other *A. xylosoxidans* strains present in the NCBI GenBank database. Specifically, it clusters within a clade supported by a 93% bootstrap value along with *A. xylosoxidans* strains and *Achromobacter insuavis*. The isolate is more distantly related to another clade formed by the strains with accession codes: *Achromobacter xylosoxidans* with a bootstrap value of 96%. This confirms that isolate H11_contig_1 belongs to the *A. xylosoxidans* species complex, but also is closely related to strains within the species. While this finding supports the identification of *A. xylosoxidans* as a carrier of quinolone resistance genes in fish farming environment.

DISCUSSION

The present study investigated the prevalence of quinolone resistance in bacteria isolated from freshwater fish farming sites in Southern Punjab, Pakistan. The overall purpose was to assess the current antibiotic resistance landscape within this important aquaculture region. The main findings revealed a concerning level of phenotypic resistance to enrofloxacin, ciprofloxacin, and norfloxacin, with significant variation across districts and between water and sediment samples. Furthermore, molecular analysis confirmed the presence of genes (*qnrA*, *qnrB*, *qnrS*) in phenotypically resistant isolates, with *qnrS* being the most frequently detected gene. Species identification demonstrated *Pseudomonas aeruginosa* and *Achromobacter xylosoxidans* as carriers of quinolone resistance determinants.

The observed phenotypic resistance levels align with findings from other studies highlighting the growing problem of AMR in aquaculture. Omotoso et al. (2025) reported resistance to ciprofloxacin and other antibiotics in bacteria isolated from fish ponds in Nigeria, while Mendoza and Reyes (2024) found high resistance to ampicillin, rifampicin, and erythromycin in bacteria from aquaculture sites in the Philippines. These results, along with those of the current study, suggest that antibiotic resistance in aquaculture is widespread phenomenon. Ali (2025) also observed resistance to ciprofloxacin among various bacterial species isolated from frozen fish in Iraq, further emphasizing the public health implications of AMR in aquaculture.

The observed district-wise and source-specific variations in quinolone resistance are particularly noteworthy. The high enrofloxacin resistance in Multan water samples and Bahawalpur sediment, contrasted with its absence in Muzaffargarh, suggests that local farm management practices, intensity of aquaculture, specific antimicrobial usage patterns, or even differences in source water quality may play a significant role in shaping local resistance landscapes. Similar geographical variations in antibiotic resistance have been reported in aquaculture systems elsewhere, often attributed to differing levels of antibiotic consumption and environmental contamination (Peng et al., 2024; Mendoza and Reyes, 2024). The higher resistance sometimes observed in sediment compared to water, as seen with enrofloxacin and ciprofloxacin in Bahawalpur, aligns with findings that sediments can act as reservoirs for antibiotic residues and resistant bacteria, potentially leading to prolonged exposure and selection (Prescott and Barkovskii, 2022).

In this study the observed phenotypic resistance to enrofloxacin, ciprofloxacin, and norfloxacin is consistent with the resistance patterns reported by Manzoor et al. (2023) in *Edwardsiella tarda* from tilapia farms in Punjab, Pakistan, where resistance to amoxicillin, erythromycin, and flumequine was prevalent. Furthermore, Qamar et al. (2025) also reported a high prevalence of *Escherichia coli* and *Klebsiella pneumoniae* in fish samples from Faisalabad, Pakistan, with associated *qnr* genes, which aligns with the detection of *qnr* genes in quinolone-resistant isolates in the current study. In line with Ullah et al. (2023), antibiotic resistance is prevalent and has many diverse gene profiles. This growing evidence of AMR is concerning and needs to be considered in future policy implementation.

The molecular investigation into resistance mechanisms further elucidates the genetic basis of the observed phenotypic resistance. The frequent detection of PMQR genes, particularly *qnrS* (37-40%), followed by *qnrA* (27-33%), is consistent with global trends

indicating the widespread dissemination of these genes. MAR index value greater than 0.2 in the current study suggests contamination due to anthropogenic antimicrobial usage, while values below 0.2 indicate minimal or no exposure to these drugs. MAR index (0.5) with the range reported in a key study from Pakistani aquaculture (Shah et al., 2012), demonstrating that our findings are consistent with previous work in the country. *qnrS* and *qnrA* are among the most commonly reported PMQR genes in *Enterobacteriaceae* and other Gram-negative bacteria from both clinical and environmental sources, including aquaculture (Röderova et al., 2017; Wang et al., 2024). We have revised the Discussion to include a direct comparison of our observed MAR index (0.5) with the range reported in a key study from Pakistani aquaculture (Shah et al., 2012), demonstrating that our findings are consistent with previous work in the country.

The presence of these PMQR genes in a significant portion of resistant isolates from Southern Punjab fish farms suggests a considerable potential for horizontal gene transfer, which can facilitate the rapid spread of quinolone resistance among diverse bacterial populations (Tapia-Cornejo et al., 2024). While PMQR genes typically confer low-level resistance, their co-occurrence with chromosomal mutations, such as those in *gyrA* and *gyrB*, can lead to clinically significant high-level resistance (Bhatt and Chatterjee, 2022). The prevalence of PMQR genes in aquatic environments, especially those impacted by aquaculture and potentially hospital effluents, has been highlighted in other Asian regions, underscoring the role of these environments as melting pots for resistance determinants (Röderova et al., 2017; Mahmud et al., 2018).

The detection of PMQR genes in quinolone-resistant isolates confirms the presence of plasmid-mediated antimicrobial resistance in Southern Punjab fish farms. Similar findings have been reported in other studies (Dhanapala et al., 2021), emphasizing the importance of horizontal gene transfer in the dissemination of AMR. The identification of *P. aeruginosa* and *A. xylosoxidans* as carriers of quinolone resistance determinants adds to the growing list of bacterial species implicated in AMR in aquaculture environments. Similar to the findings by Duman et al. (2021) and Ndegwa et al. (2025) this demonstrates the importance of understanding pathogen virulence so control measure strategies can be more effective, especially since as Lee et al. (2023) also reported, pathogens such as *S. parauberis* are continuously evolving. Furthermore, El-Gohary et al. (2020) highlight that antibiotic stewardship programs also need to take into account seasonal variation and the effects that this might have on pathogenic species. The presence of *P. aeruginosa* and *A. xylosoxidans* species carrying quinolone resistance genes in fish farm water and sediment highlights a potential risk for fish health (complicating treatment of *Pseudomonas* infections) and a potential zoonotic concern, as these bacteria can colonize or infect humans. This underscores the importance of viewing aquaculture environments not just as food production units but also as potential reservoirs for clinically relevant resistant pathogens (Wanja et al., 2019).

The widespread occurrence of PMQR genes detected in this study warrants particular attention, given their well-documented role in the horizontal dissemination of resistance. While the presence of PMQR genes has been extensively reported in clinical settings and, increasingly, in livestock and environmental samples globally (Alhomoud et al., 2017; Kadry et al., 2024), comprehensive data from Pakistani aquaculture environments, specifically focusing on environmental isolates from water and sediment, has been notably deficient. Previous work in South-East Asia, including Pakistan and Bangladesh, has identified PMQR genes in *Enterobacteriaceae* from human infections and poultry (Mahmud et al., 2018), but the direct linkage to and prevalence within the aquatic farm environment itself remained less clear. This study begins to fill that critical knowledge gap by demonstrating that genes like *qnrS* and *qnrA* are indeed prevalent within the background microbiota of freshwater fish farms in Southern Punjab. This finding is crucial because these environmental bacteria can serve as a reservoir from which PMQR genes can be acquired by fish pathogens or even human-associated bacteria, especially in regions where water resources are shared or where fish farm effluent may impact surrounding ecosystems. The relatively high consumption of quinolones reported in humans in the wider Middle East and North Africa region, often in settings with less stringent antimicrobial use regulations (Yassine et al., 2019; Gilham et al., 2024), further underscores the potential for complex interactions and bidirectional transfer of resistance determinants between human, animal, and environmental sectors.

The study highlights the critical need for surveillance and ongoing assessment of antibiotic resistance profiles, as Ismail et al. (2024), and Que et al. (2022) both reported, this will allow for management protocols to be more reliable to prevent upsurges of disease. Furthermore, Wanja et al. (2019) as well as Teerakun et al. (2025) both highlighted the role of water quality in the aquaculture system, and thus should also be considered in terms of how antibiotics can be utilized to prevent virulence. Moreover, it is vital to remember the critical role of appropriate testing identification as reported by Foysal et al. (2024) who demonstrated the differences in rural vs. urban environments, so that the right testing methods can be utilized for effective monitoring and mitigation. Finally, as suggested by Testerman et al. (2022) further research should be conducted in a variety of aquaculture environments, as this will help with generating effective disease husbandry practices. This also echoes the need for further investigation of different bacterial communities, as pointed out by Hadzevych et al. (2022). Our molecular screening for genetic determinants targeted only the classical *qnrA, qnrB*, and *qnrS* gene families and did not extend to other important plasmid-mediated mechanisms such as the *aac(6')-Ib-cr* gene or efflux pumps like *qepA*. Furthermore, while our species identification confirmed the presence of clinically relevant pathogens. However, there is a need for future research employing broader molecular screening panels or whole-genome sequencing to fully characterize the quinolone resistome and the diversity of its bacterial hosts in these aquaculture systems.

Conclusions: This study provides compelling evidence of the prevalence and genetic basis of quinolone resistance in bacteria inhabiting freshwater fish major aquaculture

districts (Bahawalpur, Lodhran, Multan, Muzaffargarh) Southern Punjab, Pakistan. The high levels of phenotypic resistance to enrofloxacin, ciprofloxacin, and norfloxacin, coupled with the frequent detection of plasmid-mediated (*qnrS, qnrA*) resistance genes, highlight these aquaculture systems as significant environmental reservoirs of quinolone resistance. The substantial district-wise variations in resistance patterns further underscore the influence of local farming practices and environmental factors on the dissemination of antimicrobial resistance in the region. The presence of multidrug-resistant bacteria, including opportunistic pathogens like *P. aeruginosa* and *A. xylosoxidans*, underscores the potential for both animal health challenges within aquaculture and potential risks to public health. These findings, while specific to the unique ecological and agricultural context of Southern Punjab, contribute valuable knowledge to the growing global concern regarding antimicrobial resistance in aquaculture. Based on these findings, we recommend the following specific actions for Pakistan: (1) Development and implementation of national guidelines for the prudent use of antibiotics particularly quinolones in aquaculture and restricting their prophylactic use; (2) Enhancement of on-farm biosecurity protocols, including water source management and disinfection to reduce disease incidence; (3) Integration of environmental AMR surveillance, focusing on key resistance genes like *qnrS*, and (4) the development of National AMR Surveillance Strategy for Aquaculture to monitor trends of antibiotic use and suggestions for the policy makers.

Authors contribution: SR: SQAS; designed study, executed experiment, analyzed data, involved in writing manuscript. HN: analyzed data, involved in reviewed manuscript. ASA: designed study, executed experiment, analyzed data, involved in review & submission of manuscript

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