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RESEARCH ARTICLE

Prevalence, antimicrobial profiling and molecular characterization of antimicrobial resistant genes of pathogenic bacteria detected in *Channa marulius* of the Indus riverine system in Pakistan

Shahid Mahmood[1], Fayyaz Rasool[2*], Shakeela Parveen[3], Amina Ayub[4], Kashif Manzoor[1], Matiullah[1], Muhammad Danish Latif[1], Talib Hussain[5], Aafaq Younas[1], Ghulam Rabbani[6], Nigah Hidait[6] and Khalid Mahmood Anjum[7]

[1]Department of Fisheries and Aquaculture, University of Veterinary and Animal Sciences, Lahore 54600, Pakistan
[2]Department of Zoology, Faisalabad Campus, University of Education, Lahore 54590, Pakistan; [3]Department of Zoology, Wildlife and Fisheries. University of Agriculture Faisalabad 38000, Pakistan; [4]Department of Zoology, Wildlife and Fisheries. Sub Campus Depalpur-Okara, University of Agriculture Faisalabad 38000, Pakistan; [5]Department of Zoology, Government College University Lahore, 54000, Pakistan; [6]Department of Zoology, University of Veterinary and Animal Sciences, Lahore 54600, Pakistan; [7]Department of Wildlife and Ecology, University of Veterinary and Animal Sciences, Lahore 54600, Pakistan
*Corresponding author: fayyaz.rasool@ue.edu.pk

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ABSTRACT

Channa marulius is a freshwater fish of riverine systems known for its economic and ecological importance that has recently been introduced into the farming system. Antibiotic resistance genes reduce the effectiveness of treatments in both public health and aquaculture, leading to resistant pathogens, economic losses, and significant threats to human health and environmental sustainability. The current study aimed to identify antibiotic resistance (ABR) genes in five selected bacterial species and their prevalence in *C. marulius* sampled from riverine system in Pakistan. Samples were collected from different organs of 480 fish samples of *C. marulius*. DNA was isolated and ABR genes were identified in the selected bacteria through PCR amplification. Phylogenetic relationship among selected bacteria was compared by phylogenetic tree of *gyrB* and *16S rRNA* gene. Antimicrobial susceptibility was tested against 14 antibiotic discs. A total of 135 (28%) including 29 (6.0%) *E. tarda*, 33 (6.9%) *E. coli*, 31 (6.4%) *A. hydrophila*, 23 (4.8%) *F. columnare*, and 19 (3.9%) *S. aureus* isolates, were retrieved. Phylogenetic tree analysis revealed 100% similarity between *S. aureus* and *F. columnare* while 90% among *A. hydrophila*, *E. coli*, and *E. tarda*. Maximum 5.62% occurrence of *sul3* gene was recorded in *E. tarda*, 6.46% of *qnrA* in *F. columnare*, 5.42% of bla_{TEM} in *E. tarda*, and 6.25% of *tetA* in *E. coli*. Finally, it was concluded that introduction of wastewater from different industries into rivers causes emergence of ABR genes in pathogenic bacteria which can increase risk of infection and transfer ABR genes to other bacteria.

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INTRODUCTION

Rapid expansion of aquaculture sector worldwide is an excellent source of high-quality food products, economic benefit, job opportunities (Boyd *et al*., 2022), life-sustaining animal proteins and nutrients. Fish and its valuable products have played a major role to overcome food insecurity caused by rapidly increasing human population. Hence, fish protein has become a vital component of a sustainable diet in the future (Froehlich *et al*., 2018). Global average fish consumption is 20.5kg per capita. Fish is a low-cost protein source, second only to meat, providing 60% of the world's protein intake and serving as a primary protein supplement in several countries (FAO, 2020).

Channa marulius, also known as the giant snakehead, is a freshwater fish that holds significant commercial value. It is widely distributed across various aquatic habitats such

as canals, lakes, marshes, ponds, reservoirs, rivers, swamps, and rice fields in countries such as Cambodia, China, India, Pakistan, and Thailand (Rahman *et al*., 2013). *C. marulius* is commonly known as 'Sole' and it belongs to the Channidae family (Nelson *et al*., 2016). The high-quality meat yield, longer shelf life, and high market demand make *C. marulius* a valuable species. It is also well-suited for intensive rearing systems due to its ability to adapt to confinement, fast growth rates, high survival rate, and efficient feed conversion ratio (Nazir *et al*., 2022). Three species of Channa, including *C. marulius*, *C. punctata*, and *C. striata*, are naturally found in Pakistan's riverine systems, but they are not commercially farmed. *C. marulius* has been recently introduced for farming purposes in Pakistan. This species is particularly appealing due to its larger size, which can reach up to 30 kg, and is considered an attractive alternative to other snakehead species (Adamson and Britz, 2018; Ruber *et al*., 2020).

Fish diseases caused by various pathogenic microorganisms including bacteria, viruses, parasites, and protozoa are a major challenge in aquaculture (Jassim *et al*., 2019). Bacteria that are naturally resistant in both land and water environment can easily transfer antibiotic resistance (ABR) genes to harmful bacteria in fish due to their close interaction (Cantas *et al*., 2013). So, fish spread antibiotic resistant bacteria and their ABR genes, promoting their propagation (Marti *et al*., 2014). Fish farmers utilize a wide variety of antibiotics to tackle mortality in fish that is caused by antibiotic-resistant bacteria (Schar *et al*., 2020). In aquaculture, the indiscriminate and excessive use of antibiotics contributes to the emergence of AMR bacteria and their genes (Cabello *et al*., 2016). Antibiotic-resistant bacteria pose a growing and significant public health threat as they can employ genetic mechanisms to develop resistance to antibiotics (Sun *et al*., 2022). Studying ABR genes in fish bacteria is essential for sustainable aquaculture, ensuring healthier fish populations, and preventing the spread of antibiotic-resistant pathogens (Aslam *et al*., 2021; Ahmad *et al*., 2023), which supports food safety and the global "One Health" approach to combat antimicrobial resistance (Cella *et al*., 2023).

The current study was conducted to detect ABR genes in pathogenic bacteria isolated from *C. marulius* and analyze antimicrobial susceptibility testing. Phylogenetic tree analysis was performed to check phylogenetic relationship amidst isolated bacteria.

MATERIALS AND METHODS

Fish sampling and ethical approval: A total of 480 *Channa marulius* samples were randomly collected from April 2022 to December 2022 from Head Baloki, Head Chashma, Head Taunsa, and Head Trimmu, located within the riverine system of Punjab, Pakistan. The fish samples were packed in ice-treated containers and transported to the laboratory at the Department of Zoology, University of Education Lahore (Faisalabad Campus), Pakistan. All protocols were approved by the Advanced Studies and Research Board (ASRB) of the University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan (reference number: DAS/358, 02-03-2023). A GIS map of sampling sites is shown in Fig. 1.

Isolation, phenotypic, morphological, and biochemical characterization of bacteria: Overall, 135 fish samples were disinfected, and swabs were randomly collected from suspected organs (skin, stomach, kidney, liver, intestine, spleen, and gills). The swabs were inoculated and streaked onto trypticase soy agar (TSA) media plates (LAB, UK) and incubated at 37°C overnight. A pure culture of bacterial isolates was obtained by inoculating a few colonies from the freshly cultured media onto new TSA media plates, which were then incubated at 37°C for 24 hours (Lima *et al*., 2008). Bacterial isolates were

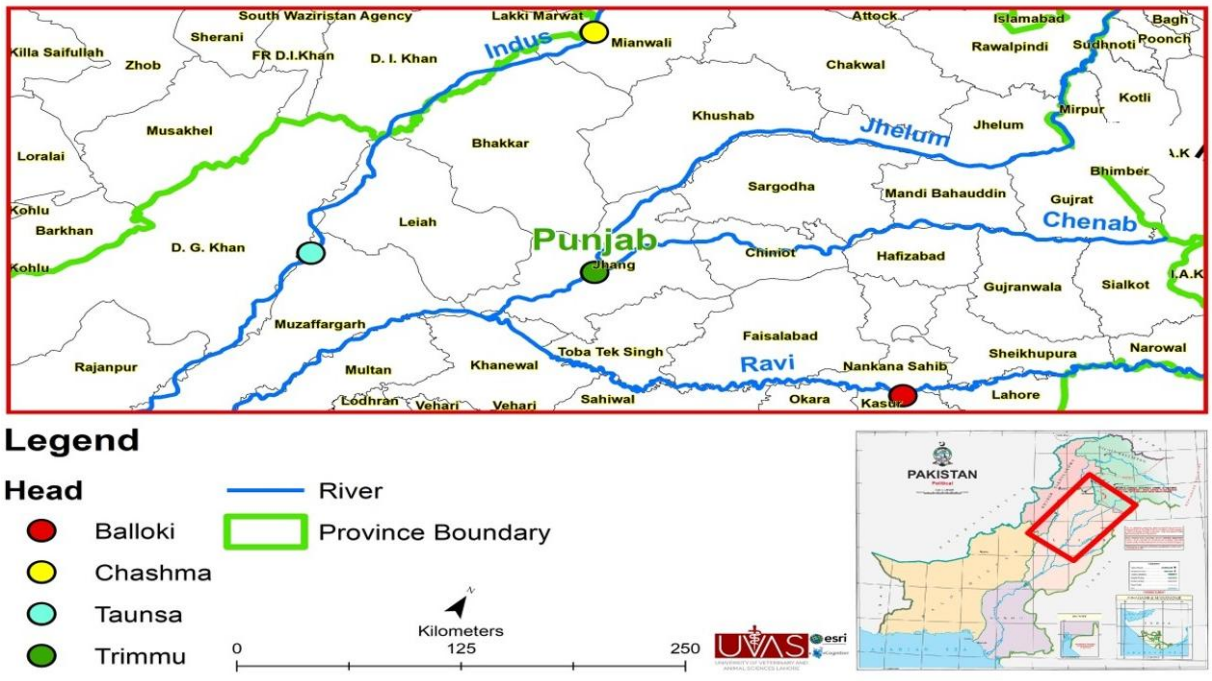

Fig. 1: GIS map of sampling sites.

subjected to phenotypic, morphological, and biochemical characterization, including Gram staining, motility, catalase, oxidase, glucose, sucrose, lactose, indole production, H_2S production, and urease test (Xiao *et al*., 2008).

DNA extraction: The DNA of bacterial isolates was extracted using the GeneJET Genomic DNA Purification Kit (Thermo Scientific, USA) and quantified on a 1% agarose gel stained with ethidium bromide (EtBr) using a standard molecular marker by gel electrophoresis. The isolated DNA was stored at -20°C for further use.

Molecular identification of an ABR genes and phylogenetic tree analysis of *gyrB* and *16S rRNA* genes of bacteria: *16S rRNA*, *gyrB*, and antibiotic resistance genes, including *sul3*, *qnrA*, bla_{TEM}, and *tetA*, were amplified by PCR (Table 1). The amplified PCR products were quantified on a 1% agarose gel stained with ethidium bromide (EtBr) and compared with a 1 KB DNA ladder (molecular marker). PCR products of all amplified genes were sequenced using Sanger's method at BGI Hong Kong Company Limited, China (Wang *et al*., 2016). The partial sequences were compared for taxonomic characterization using NCBI BLAST and submitted to the GenBank database. Phylogenetic tree analysis of the *16S rRNA* and *gyrB* genes of bacteria was constructed using the bootstrap method in MEGA 11.0 (Shah *et al*., 2009).

Antimicrobial susceptibility testing of bacteria: Antimicrobial sensitivity testing of bacteria was conducted using the Kirby-Bauer disc diffusion method on Mueller-Hinton (MH) agar plates with twelve antibiotics. The MH agar plates were incubated for 24 hours at 28°C. Reference strains used as controls were ATCC 25922 for *E. coli*, ATCC 25923 for *S. aureus*, ATCC 49512 for *F. columnare*, ATCC 7966 for *A. hydrophila*, and ATCC 15947 for *E. tarda*. Diameter of the inhibition zone were measured and interpreted to classify bacteria as resistant, moderately susceptible, and susceptible (CLSI, 2020).

Statistical analysis: The prevalence/occurrence of bacteria was compared using a chi-square test of independence with respect to fish sex, sampling site, organs, and season. Descriptive statistics such as proportions and frequency were employed in summarizing the data.

RESULTS

Biochemical identification of bacteria: Biochemical characterization identified *E. tarda*, *E. coli*, *A. hydrophila*, and *F. columnare* as Gram-negative, and *S. aureus* as Gram-positive. *E. tarda* and *A. hydrophila* tested positive for H_2S production, urease, and indole, with *A. hydrophila* also positive for oxidase, sucrose, and lactose. *S. aureus* was positive for sucrose, and *E. coli* for lactose and indole. All isolates were positive for catalase and glucose.

Occurrence of antibiotic resistance genes of bacteria: ABR genes of bacteria were amplified by PCR. Maximum 5.62% occurrence of *sul3* gene was recorded in *E. tarda*, 6.46% of *qnrA* in *F. columnare*, 5.42% of bla_{TEM} in *E. tarda*, and 6.25% of *tetA* in *E. coli*. Occurrence of AMR genes and accession numbers allotted by NCBI against all identified genes are given in Table 3. Chi-square test of independence showed insignificant difference ($P>0.05$) in occurrence of antibiotic resistance (ABR) genes (Table 4).

Prevalence of bacteria: Maximum prevalence of 5.42%, 6.25%, 6.87%, 6.25%, 4.79%, 5.8%, and 5.21% was recorded in skin, liver, intestine, stomach, gills, kidney, and spleen of infected *C. marulius* respectively. Maximum prevalence of 12.5%, 9.17%, 5%, and 4.17% was recorded in fish samples collected from Baloki, Trimmu, Taunsa, and Chashma respectively. Pathogenic bacteria infected 16.46% female fish as compared to 13.75% male fish. Maximum seasonal prevalence of 15.21%, 9.79%, and 3.12% was recorded in summer, autumn, and winter respectively (Table 2). A total of 135 (28.12%) isolates including 29 (6.0%) *E. tarda*, 33 (6.9%) *E. coli*, 31 (6.4%) *A. hydrophila*, 23 (4.8%) *F. columnare*, and 19 (3.9%) *S. aureus* isolates, were retrieved.

Antimicrobial susceptibility test: All bacterial isolates showed resistance to amoxicillin, ampicillin, neomycin, and norfloxacin, while sensitive to gentamicin,

Table 1: Primers sequence and conditions for amplification of antibiotic reistance (ABR) genes, *gyrB* and *16S rRNA* gene by PCR.

Target Gene	Primer sequence (5-3)		Target bp size	Cycles	Annealing	References
16S rRNA	F	AGAGTTTGATCCTGGCTCAG	1503	30	52°C for 1min	Wimalasena *et al.* (2017)
	R	ACGGCTACCTTGTTACGACTT				
tetA	F	GCTACATCC TGCTTGCCTTC	813	35	55°C for 1min	
	R	CATAGATCGCCGTGAAGAGG				
bla_{TEM}	F	CATTTCCGTGTCGCCCTTATTC	873	35	55°C for 90 s	
	R	CGTTCATCCATAGTTGCCTGAC				
qnrA	F	ATTTCTCACGCCAGGATTTG	654	30	60°C for 1 min	
	R	GATCGGCAAAGGTTAGGTCA				
sul3	F	AGATGTGATTGATTTGGGAGC	444	35	54.2°C for 30 s	
	R	TAGTTGTTTCTGGATTAGAGCCT				
gyrB (*E. coli*)	F	GAAGTCATCATGACCGTTCTGCA	1258	30	52°C for 1min	Francis *et al.* (2022)
	R	AGCAGGGTACGGATGTGCGAGCC				
gyrB (*E. tarda*)	F	GCGGAGATTTTGCTCTTCTT	414	35	55°C for 1min	Manzoor *et al.* (2023b)
	R	GATCGGCAAAGGTTAGGTCA				
gyrB (*A. hydrophila*)	F	GAGGACTACAGCAAGAAGGCCA	1124	35	55°C for 90 s	Wang *et al.* (2016)
	R	GACTTGGCCTTCTTGCTGTAGTC				
gyrB (*F. columnare*)	F	GAAGTCATCATGACCGTTCTGCA	411	30	60°C for 1 min	Elgendy *et al.* (2022)
	R	AGCAGGGTACGGATGTGCGAGCC				
gyrB (*S. aureus*)	F	GCGGAGATTTTGCTCTTCTT	990	35	54.2°C for 30 s	Niakhalili *et al.* (2023)
	R	GATCGGCAAAGGTTAGGTCA				

Table 2: Prevalence of bacteria with respect to sampling sites, sex and, seasons and fish organs

Bacterium	Sampling Sites				Sex		Seasons			fish organs						
	Baloki	Trimmu	Taunsa	Chashma	Male	Female	Summer	Autumn	Winter	Skin	Liver	ntestine	Stomach	Gills	Kidney	Spleen
E. tarda	12 (10%)	9 (7.5%)	5 (4.17%)	3 (2.5%)	18 (8%)	11 (4.31%)	15 (5.36%)	10 (8.33%)	4 (5%)	20 (4.17%)	23 (4.79%)	29 (6.04%)	27 (5.62%)	18 (3.75%)	26 (5.42%)	21 (4.37%)
E coli	15 (12.5%)	8 (6.67%)	6 (5%)	4 (3.33%)	10 (4.44%)	23 (9.02%)	18 (6.42%)	12 (10%)	3 (3.75%)	26 (5.42%)	30(6.25%)	33 (6.87%)	30 (6.25%)	23 (4.79%)	28 (5.8%)	25 (5.21%)
A.hydrophila	13 (10.83%)	11 (9.17%)	5 (4.17%)	2 (1.67%)	22 (9.78%)	9 (3.53%)	16 (5.71%)	10 (8.33%)	5 (6.25%)	24 (5%)	25 (5.21%)	31 (6.46%)	29 (6.04%)	23 (4.79%)	27 (5.62%)	22 (4.58%)
F.columnare	10 (8.33%)	6 (5%)	2 (1.67%)	5 (4.17%)	7 (3.11%)	16 (6.27%)	13 (4.64%)	8 (6.67%)	2 (2.5%)	19 (3.94%)	17 (3.54%)	23 (4.79%)	19 (3.96%)	16 (3.33%)	18 (3.75%)	17 (3.54%)
S. aureus	8 (6.67%)	4 (3.33%)	6 (5%)	1 (0.83%)	9 (4%)	20 (7.84%)	11 (3.93%)	7 (5.83%)	1 (1.25%)	15 (3.12%)	14 (2.92%)	19 (3.96%)	16 (3.33%)	12 (2.5%)	14 (2.92%)	15 (3.12%)

Table 3: Occurrence of antibiotic resistance (ABR) genes, *gyrB*, *16S rRNA* gene and accession numbers of bacteria

Bacterium	antibiotic resistance (ABR) genes, *gyrB* and *16S rRNA* gene						Accession numbers					
	qnrA	*bla*$_{TEM}$	*tetA*	*sul3*	*gyrB*	*16S rRNA*	*qnrA*	*bla*$_{TEM}$	*tetA*	*sul3*	*gyrB*	*16S rRNA*
E. tarda	29 (6.04%)	26 (5.42%)	28(5.8%)	27 (5.62%)	29 (6.04%)	29 (6.04%)	OQ729988	OQ726104	OQ718930	OQ729972	OQ699126	OQ613271
E coli	26 (5.42%)	23 (4.79%)	30 (6.25%)	25 (5.21%)	33 (6.87%)	33 (6.87%)	OQ557497	OQ536310	OQ729941	OQ729976	OQ699118	OQ613278
A. hydrophila	27 (5.62%)	19 (3.96%)	29 (6.04%)	21 (4.37%)	31 (6.46%)	31 (6.46%)	OQ710327	OQ726100	OQ790132	OQ729968	OQ557501	OQ613281
F. columnare	31 (6.46%)	16 (3.33%)	22 (4.58%)	24 (5%)	23 (4.79%)	23 (4.79%)	OQ729996	OQ726096	OQ790130	OQ729984	OQ699122	OQ691648
S. aureus	24 (5%)	20 (4.17%)	26 (5.42%)	26 (5.42%)	29 (6.04%)	29 (6.04%)	OQ729992	OQ726092	OQ790131	OQ729980	OQ710323	OQ695458

Table 4: Results of statistical analysis; chi-square test of independence showing X^2-value and P-value with respect to parameters.

Parameter	Chi-squared value	p-value
Organs	108.5	0.628^{ns}
Bacterial Species	88.3	0.158^{ns}
Sampling Sites	37.3	0.408^{ns}
Fish Sex	8.0	0.433^{ns}
Seasons	30.0	0.268^{ns}
Occurrence of ABR Genes	36.0	0.607^{ns}

ns = non-significant result.

chloramphenicol and tetracycline and intermediate resistance against cefotaxime. *A. hydrophila*, *E. tarda*, *E. coli*, and *F. columnare* showed resistance to sulfamethoxazole. *E. tarda*, *S. aureus*, and *F. columnare* showed intermediate resistance against ciprofloxacin. *A. hydrophila* isolates showed intermediate resistance against streptomycin.

Phylogenetic tree analysis: Phylogenetic trees were constructed using MEGA 11.0, incorporating multiple sequence alignment, model selection, and distance-based methods (neighbor-joining method) analysis. Phylogenetic tree of *16S rRNA* gene of bacteria revealed 100% similarity among *F. columnare*, *A. hydrophila*, and *S. aureus* while 90% similarity among *E. tarda* and *E. coli* strains. Phylogenetic tree analysis of *gyrB* gene of *E. tarda*, *E coli*, and *F. columnare* revealed 100% similarity with other strains isolated in previous studies, while phylogenetic tree analysis of *A. hydrophila* and *S. aureus* showed 97% and 98% similarity respectively with other strains isolated in previous studies. Phylogenetic trees of *16S rRNA* and *gyrB* gene are shown in Fig. 2 and 3.

DISCUSSION

Native to riverine systems, *C. marulius* faces a persistent threat to its population due to the deterioration of water quality caused by the discharge of wastewater from pharmaceutical and chemical industries containing multiple antibiotics and other chemicals (Batool *et al*., 2021; Farid *et al*., 2021). The continuous exposure of wastewater containing antibiotics leads to the emergence of multiple antibiotic resistance genes in pathogenic bacteria that target *C. marulius* (Hussain *et al*., 2019). Industrial antibiotics and pollutants promote the gene transfer and survival among resistant bacteria. This accelerates the emergence and dissemination of ABR in aquatic environments (Barathan *et al*., 2024; Wu-Wu *et al*., 2024). Antimicrobial use (AMU) in food animals in Pakistan, also significantly contributes to the spread of antimicrobial resistance (AMR) in human health, aquaculture, and other animal populations. This occurs through the transfer of resistant bacteria and genes via shared environments like water and soil. Addressing AMU in food animals is vital for a comprehensive approach to combating AMR across all sectors (Mohsin *et al*., 2019; Umair *et al*., 2022).

In the current study, we recovered 33 (6.9%) isolates of *E. coli* in fish sampled at all sites. A recent study reported 61.1% prevalence of *E. coli* isolates in Cochin, India (Francis *et al*., 2022). Another study reported 56.7% prevalence of *E. coli* in fish farms in Thailand (Thaotumpitak *et al*., 2022). Similarly, 50% isolates of *E. coli* were reported in fish of riverine system in Northern part of Bangladesh (Faridullah *et al*., 2022). Dewi *et al*. (2022) reported 44.5% prevalence in fish farms on the West Coast of Peninsular Malaysia. In another recent study Akter *et al*. (2022) recovered 90% isolates of *E. coli* from fish market of Dhaka city in Bangladesh that were 100% resistance to erythromycin while sensitive to norfloxacin, ciprofloxacin, gentamycin, and chloramphenicol. These differences in prevalence may be due to variations in sampling methods, geographical locations, population demographics, and changes in the bacterial population over time.

F. columnare is one of the major fish pathogens that spreads Columnaris which has caused infections of skin and gills in 36 freshwater fish species. 17.5% prevalence of *F. columnare* was reported in Egyptian fish while 14 and 15% in gills and skin respectively, 20 and 40% in autumn and summer, respectively (El-Tawab *et al*., 2020). We recovered 4.8% isolates of *F. columnare*. Elgendy *et al*. (2022) also reported 60% prevalence of *F. columnare* while they recovered 44.5% and 55% isolates from gills and skin of infected fish respectively in Egypt. Diyie *et al*. (2022) also recovered 22% isolates of *F. columnare* in

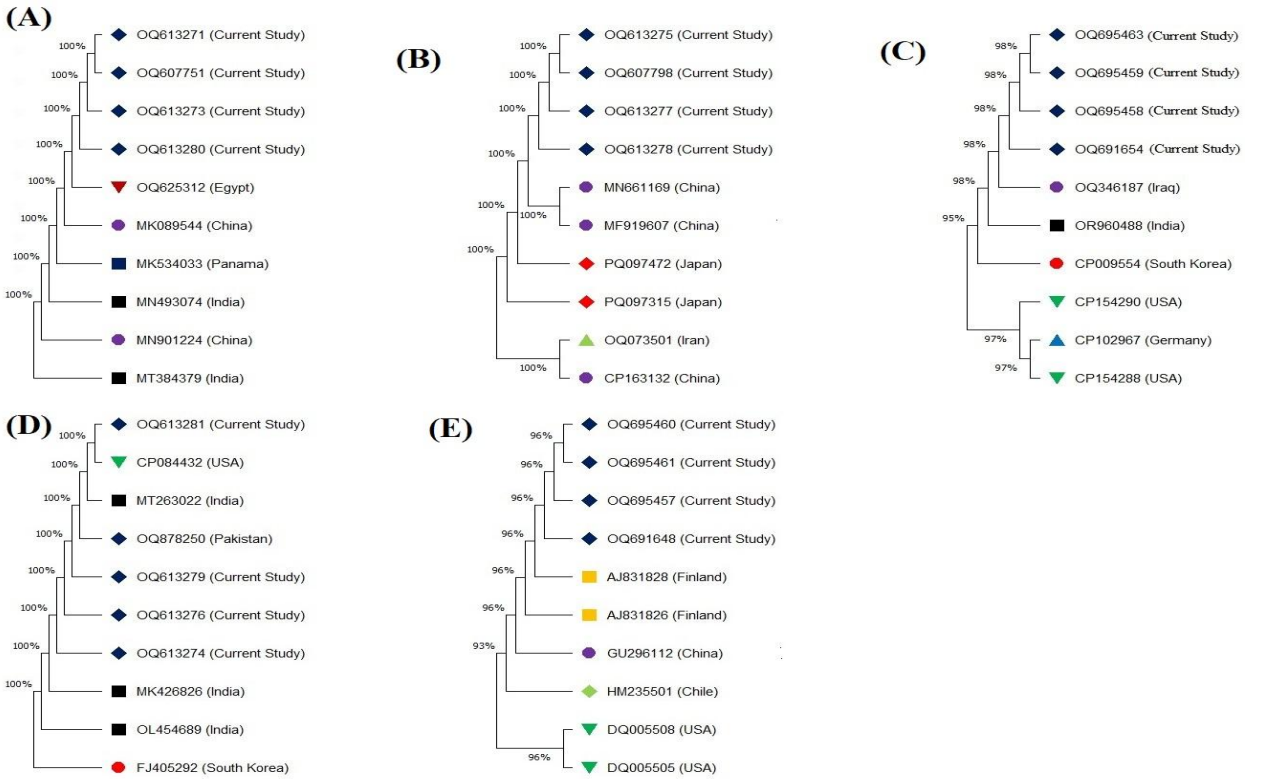

Fig. 2: *Phylogenetic tree of 16S rRNA gene of selected bacteria (A) A. hydrophila, (B) E. coli, (C) S aureus, (D) E. tarda, (E) F. columnare.*

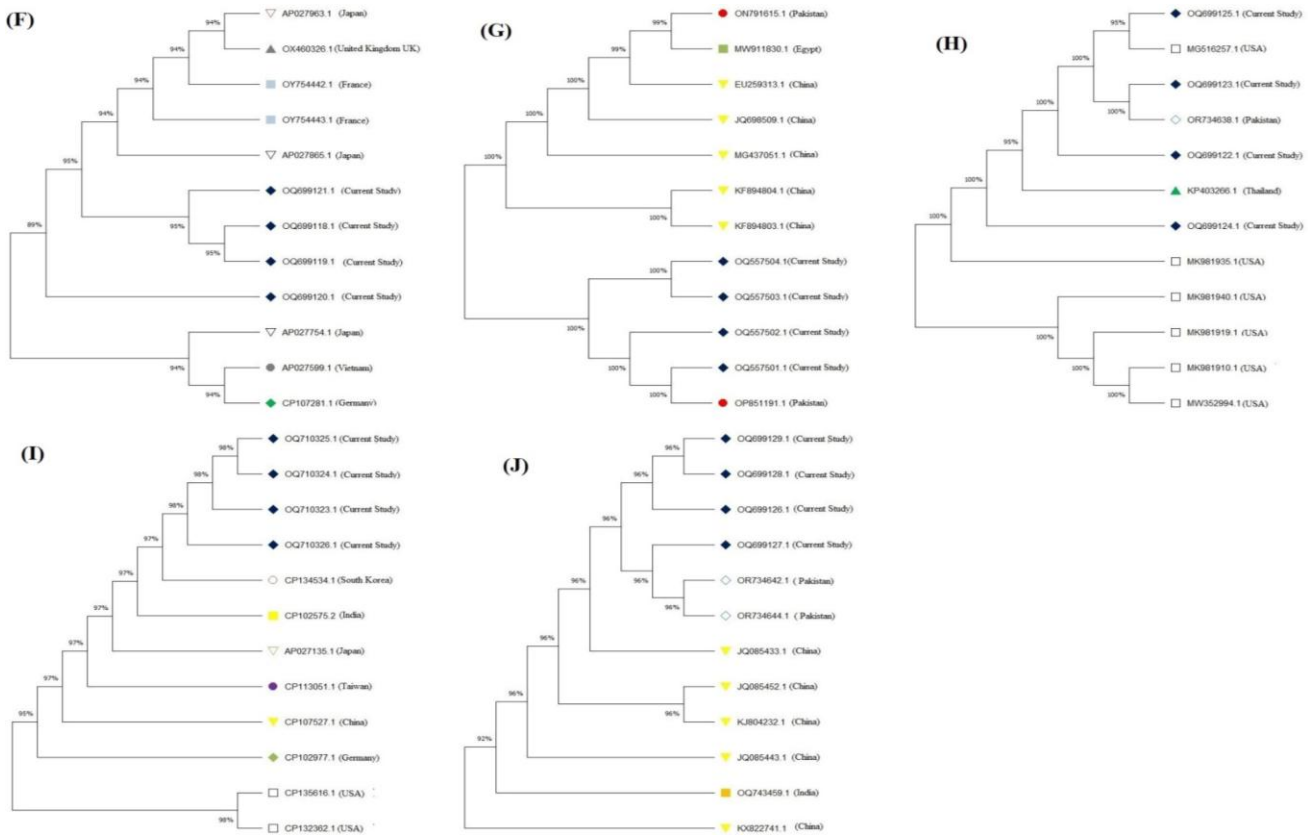

Fig. 3: *Phylogenetic tree of gyrB gene of selected bacteria (F) E. coli, (G) E. tarda, (H) F. columnare, (I) S. aureus, (J) A. hydrophila.*

Ghana. *A. hydrophila* spreads Aeromoniasis and motile Aeromonas septicemia (MAS) in fish that was resistant to ampicillin, followed by oxytetracycline and tetracycline (Thaotumpitak *et al*., 2023). El-Hossary *et al*. (2023) and Thaotumpitak *et al*. (2022) recovered 4 (1.2%) isolates and 15 isolates of *A. hydrophila* in Thailand. These variations in results may be due to differences in laboratory techniques, diagnostic criteria, patient inclusion exclusion criteria, or the emergence of new strains and antimicrobial resistance patterns.

In the current study, we recovered 3.9% isolates of *S. aureus*. Niakhalili *et al*. (2023) reported a 7.1% prevalence of *S. aureus* in fish in Iran. Sivaraman *et al*. (2021) recovered 173 *S. aureus* isolates from market fish in Assam in India. Sivaraman *et al*. (2022) also recovered 15% *S. aureus* isolates in fish in India and these isolates showed resistance gentamicin and ampicillin. In a previous study in China, researchers recovered *S. aureus* in 119 fish samples with a prevalence of 37.2% (Rong *et al*., 2017). In the current study, we recovered 29 (6.04%) isolates of *E. tarda* from sampled fish. Similarly, a recent study recovered from 147 (27.2%) *E. tarda* isolates from fish farms of Punjab in Pakistan that were sensitive to ciprofloxacin, chloramphenicol, sulfamethoxazole, gentamicin, streptomycin, and norfloxacin while resistant to flumequine, neomycin, amoxicillin, and erythromycin but intermediate resistance to cefotaxime, tetracycline, doxycycline, and ampicillin (Manzoor *et al*., 2023b; Mahmood *et al*., 2024). Similarly, in another recent study, researchers reported a prevalence of 14% of *E. tarda* in infected fish (Algammal *et al*., 2022). Whereas a recent study in Nigeria, reported a very high prevalence of 62.5% of *E. tarda* at fish farms in Ibadan (Charles *et al*., 2020). Reasons behind these variations may be study population, sampling techniques, environmental conditions, genetic mutations, and changes in antimicrobial usage patterns over time.

Horizontal gene transfer rapidly spreads ABR genes through mechanisms like transduction, transformation, and conjugation (Ahmad *et al*., 2023), increasing infection rates, resistance, and environmental impact, requiring strict antibiotic stewardship and infection control (Aslam *et al*., 2021). The spread of ABR genes in aquaculture can be mitigated by enhancing wastewater treatment with advanced filtration and biological methods, enforcing stringent antibiotic stewardship, implementing biosecurity measures, reducing stocking densities, rotating antibiotics, and promoting the use of probiotics, vaccines, and natural antimicrobials (Noman *et al*., 2021; Huang *et al*., 2021; Barathan *et al*., 2024). Moreover, farmers must be educated on responsible practices and regularly monitor antibiotic use (Caudell *et al*., 2020; Manzoor *et al*., 2023b).

Conclusions: Multiple industries (especially those which manufacture antibiotics and other drugs), introduce wastewater into rivers which causes emergence of antibiotic resistance (ABR) genes in pathogenic bacteria, that can increase risk of infection and transfer ABR genes to other non-pathogenic bacteria. There was no significant difference in prevalence of bacteria with respect to organs, sampling sites, fish sex, season and occurrence of ABR genes.

Declaration of competing interest: The authors declare that they have no known financial interests or personal relationship that could have appeared to influence the work reported in this paper.

Authors contribution: SM and FR conceived and designed the study. SM, SP executed the experiment and finalized the data. MU, TH, DL, AY, NH, GR and KM helped in sampling. KM assisted in experimentation. SM wrote the manuscript. SM and FR contributed equally to this work. All authors read and approved the final manuscript.

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