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CHARACTERIZATION OF BACTERIAL PATHOGENS ISOLATED FROM FRESHWATER FISH (*LABEO ROHITA*) FROM FRESHWATER AQUACULTURE

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ABSTRACT

Fishes are the significant source of high-quality protein, iodine, vitamins and minerals. Fishes can easily acquire pathogenic bacterial strains from water and act as the vehicle for transmission of pathogens to humans. The present study was designed to isolate bacteria that pose health risk to humans and the environment present in gills, gut and inhabited water samples of *Labeo rohita*, which was collected from different aquaculture farms in Bargarh district, Odisha. Bacteria were isolated using specific media and identification was performed based on phenotypic, biochemical tests and molecular approaches using genus-specific primers. In the present study, twelve bacterial strains have been isolated. The identification of bacterial isolates revealed that four isolates (FPA-1 to FPA-4) were identified as *Aeromonas* sp., four isolates (FPE-5 to FPE-8) as *E. coli* and the remaining four isolates (FPS-9 to FPS-12) were identified as *Staphylococcus* sp. These bacterial strains (*Aeromonas* sp., *E. coli* and *Staphylococcus* sp.) indicated the relative distribution of bacterial pathogens in fish samples, which could contribute significant threats to fish consuming communities. Unsafe fish handling and culturing might contribute to the prevalence of bacterial pathogens in fish.

Keywords: *Aeromonas*, *E. coli*, *Labeo rohita*, Pathogenic bacteria, *Staphylococcus*

INTRODUCTION

Aquaculture is the expanding food production approach in the world[1] accounting to 50% of the global seafood consumption, which is indispensable to satisfy the increasing fish demand. Seafood is vital for the human diet and an excellent source of lean protein, omega-three fatty acids and other nutrients [1]. Odisha is rich in aquatic resources with key fish-producing regions including Khurdha, Puri, Balasore, Bargarh and Sambalpur. Bargarh is one of the top fish producing regions in Odisha producing major carps like *Labeo rohita*, *Catla catla*, *Cirrhinus mrigala* and prawns such as *Macrobrachium rosenbergii* along with Indian minor carps and catfishes [2]. Fishes are vital source of high-quality protein, vitamins, minerals and iodine [3]. About sixty percentage of world protein is supplemented by fish and related products. With the growing population, the demand of fish and related as the source of protein is increasing tremendously [3,4]. However, fish are vulnerable to various bacterial diseases as evidenced by the appearance of clinical signs of bodily alterations influenced by the physical, chemical and biological factors that cause physiological changes in fishes [4].

Aquaculture is highly vulnerable to pollution *via* industrial wastes, agricultural runoff, land pollution and airborne infections. The microflora associated with freshwater fishes is acquired from environment where they are harvested by feeding. The pathogenic bacterial strains contaminate the

fish's skin, gills and digestive tract, thereby acting as carriers for the transmission of pathogenic bacteria, posing risk to human healthcare [3]. Several studies reported that the pathogenic strains associated with fish diseases mainly include *Salmonella sp.*, *Shigella sp.*, *Pseudomonas sp.*, *Escherichia coli*, *Staphylococcus aureus*, *Aeromonas sp.*, *Listeria monocytogenes*, *Clostridium sp.* and *Vibrio sp.* representing the dominating bacterial microflora of fishes [3,5].

Bacterial strains such as *E. coli* usually found in the digestive tracts of humans and animals, that cause human diseases [6]. The detection of *E. coli* in food indicates contamination mediated by entero-pathogenic and toxigenic microbes, which might lead to public health hazards [7]. Infantile diarrhoea caused by *E. coli* is a major cause of morbidity and mortality in infants and young children [8,9]. Additionally, *E. coli* is responsible for urinary tract infection (UTI), neonatal meningitis and enteritis. Besides, *Staphylococci* are commonly found in tissues, skin surfaces and foods contaminated by infected people or animals. The ability of *Staphylococci* to coagulate plasma is one of the unique features of pathogenic strains [10,11]. *Staphylococcus aureus* is the causative microbe for serious animal ailments, including ruminant mastitis, suppurative disease, arthritis, omphalitis and urinary tract infections [12]. The most common transmission routes include fomites transferring bacteria from infected to uninfected mammary glands, uncontrolled animal traffic between farms, handling and consuming contaminated foods. Besides, *Staphylococcus sp.* is responsible for fatal human diseases including abscesses, mastitis and pyoderma.

Aeromonas are prudently found in surface water, groundwater, drinking water, bottled mineral water particularly in biofilms and food products [13]. *Aeromonas sp.* has emerged a significant human pathogen linked to food-borne illness [14]. In children, *Aeromonas sp.* causes acute diarrhoea, while it causes traveller's diarrhoea in adults [15]. *Aeromonas* infections can result from traumatic injury in an aqueous setting, leading to cellulitis or wound infections [16]. Moreover, it is reported to cause septicaemia in individuals with underlying diseases like leukaemia, cirrhosis, meningitis, urinary tract infections, cancer and endocarditis [17]. In addition, *Aeromonas sp.* is given primary research importance and public health significance due to its ubiquitous distribution and opportunistic pathogenic potentials.

Keeping in view, this study was designed to evaluate the relative abundance of bacterial pathogens in Indian major carp *i.e. Labeo rohita* focusing on gills, gut and fresh water collected from aquafarms. Bacterial strains were isolated and characterized, followed by their molecular identification through PCR amplification using genus-specific primers. Studies on bacterial pathogens not only elucidate the relative abundance and distribution of bacterial pathogens in freshwater aquafarms but also substantiate the development of potent antimicrobial agents for long-term sustainable aquaculture with efficiency and economy.

MATERIALS AND METHODS

Study site and sampling

Aquafarms in four blocks (Attabira, Padampur, Sohela and Barpali) of the revenue district of Bargarh, Odisha were selected for the study. The study site experienced annual rainfall ranging from 1041.9 mm to 2193.6 mm over last 10 years (2014-2023), with an average rainfall of 1527 mm and 60.92% relative humidity. The aquafarms selected for the studies were located within the peripheral distance of 50 km from the district headquarters of Bargarh. The geotag photographs of the selected aquafarms were depicted (Figure 1).

Four aquafarms were selected for sampling following the proportionate random sampling method [18]. For this purpose, three different samples were collected from each block for the isolation and characterization of bacterial isolates such as (a) fish gill to isolate *Aeromonas sp.* and

Staphylococcus sp.; (b) fish gut to isolate *E. coli* and (c) water sample from respective aquafarms. Samples were collected aseptically and transferred to the laboratory for further analysis.

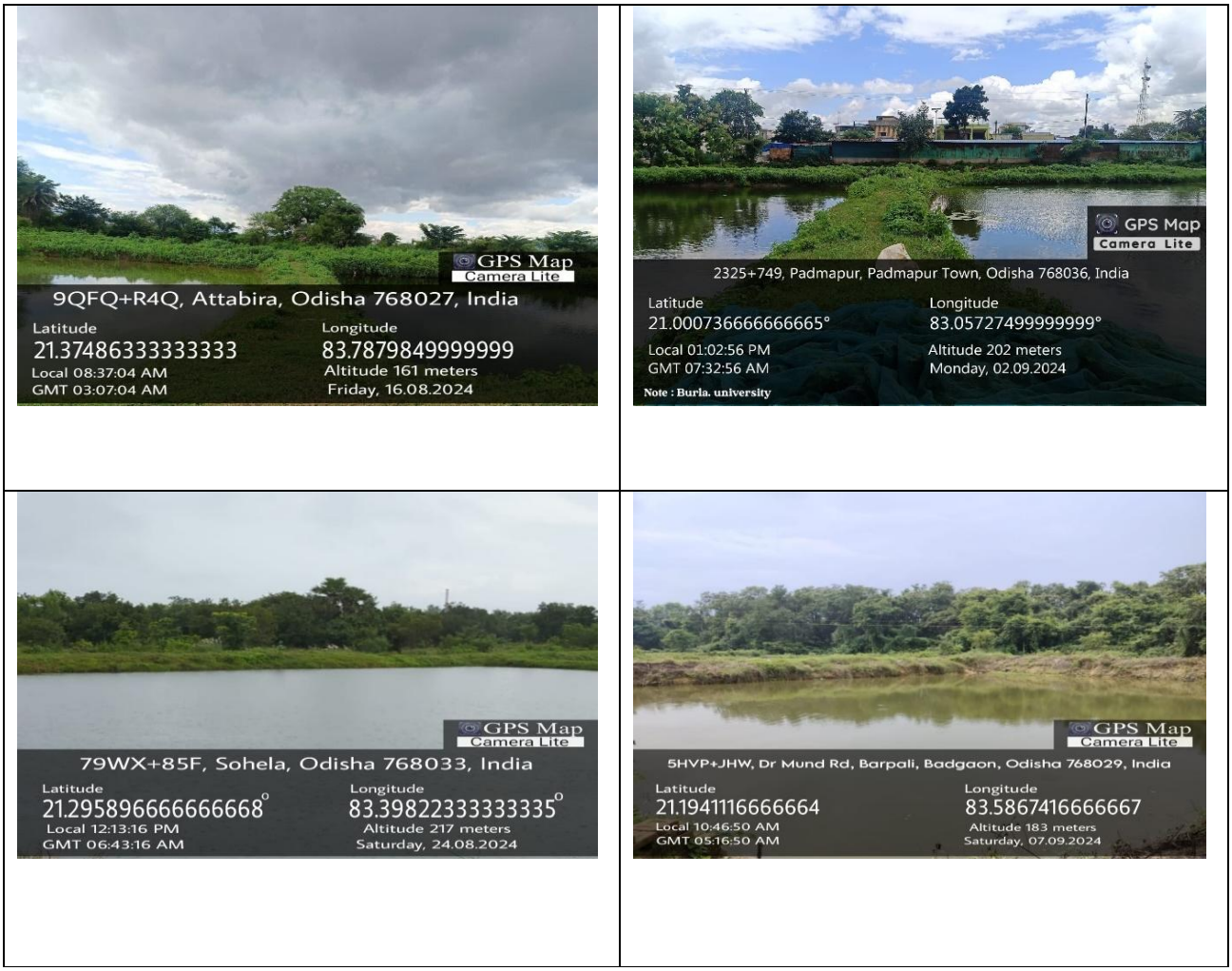


Figure 1. Geotag photographs of aquafarms selected for sampling in different blocks such as (a) Attabira; (b) Padampur; (c) Gaislet and (d) Bhatli of Bargarh district, Odisha.

Isolation of bacteria

Bacterial pathogens such as *Aeromonas sp., E. coli* and *Staphylococcus sp.* were isolated using selective media following standard microbiological methods [19]. *Aeromonas sp.* was isolated from the gill sample. The sample was inoculated using enrichment broth supplemented with alkaline peptone water and 0.5% ampicillin, incubated at 37°C for 24 hrs. A loopful culture was inoculated into ampicillin dextrin agar [ampicillin dextrin agar base+ 0.5% vancomycin+ 0.5% ampicillin] and kept at 37°C for 24 hrs. The yellow colour colony was inoculated using esculin agar and incubated at 37°C for 24 hrs. The dark-coloured colony after hydrolysis was subjected to final isolation using tryptone soya agar and incubated at 37°C for 24 hrs [20].

E. coli was isolated from the fish intestine sample. Sample was enriched with EC broth and incubated at 44°C for 24 hrs with Durham's tube. A loopful culture was inoculated in MacConkey agar. Red/pink colony was inoculated in Levine eosin-methylene blue agar and kept at 37°C for 24 hrs. Metallic sheen colony was picked for pure culture using tryptic soya agar at 37°C for 24 hrs [21]. Besides, *Staphylococcus sp.* was isolated from gill sample using tryptone soya broth supplemented with 10% sodium chloride and 1% sodium pyruvate, incubated at 37°C for 24 hrs. Streaking of culture into Baird Parker agar enriched with 5% egg yolk and 3.5% potassium tellurite

followed by incubation at 37°C for 48 hrs. The black-centred, halo-forming colony was inoculated in mannitol salt agar and incubated at 37°C for 24 hrs. The resulting yellow colony was transferred to tryptic soya agar to obtain pure culture and maintained at 37°C for 24 hrs [22].

Phenotypic characterization

Isolated colonies revealed by bacterial isolates were phenotypically characterized for their presumptive identification based on the colony morphology and Gram's stain response.

Biochemical characterization

Bacterial isolates were identified through phenotypic characterization. Different biochemical tests were conducted, including catalase, oxidase, coagulase, Voges-Proskauer, methyl red, Indole, oxidation-fermentation, citrate test, urease, triple sugar iron, carbohydrate fermentation, esculin hydrolysis and motility tests. ATCC reference strains were used as the control for comparison [19].

Molecular characterization

Genomic DNA was extracted using the Quick-DNA™ Fungal/Bacterial Miniprep kit. DNA integrity was evaluated using Nanodrop and the purity was assessed by 260/280 nm absorbance ratio with value between ~1.8 to 2.0 that indicated appreciable genomic DNA purity. Amplification of the 16S rRNA gene was performed utilizing a reaction mixture [Forward and reverse primers: 10 pmol, MgCl2: 2.5 mM, Taq DNA polymerase: 0.5 U, 200 μM each dNTPs, 1X PCR buffer (Invitrogen) and 50-100 ng DNA] using the genus-specific primers (Table 1).

Table 1. Genus specific primers including their amplicon size used for the identification of the bacterial pathogens such as *Aeromonas sp., E. coil* and *Staphylococcus sp.* isolated from different aquafarms.

Microorganisms	Gene	Genus specific primer sequence (5' →3')	Amplicon size (bp)	Reference
Aeromonas sp.	16s	F-CTACTTTTGCCGGCGAGCGG	953bp	[33]
		R-TGATTCCCGAAGGCACTCCC		
E. coli	16s	F-GTTAATACCTTTGCTCATTGA	340bp	[34]
		R-ACCAGGGTATCTAATCCTGTT		
Staphylococcus sp.	16s	F-AGCGAGTCTGAATAGGGCGTTT	108 bp	[35]
		R-CCCATCACAGCTCAGCCTTAAC		

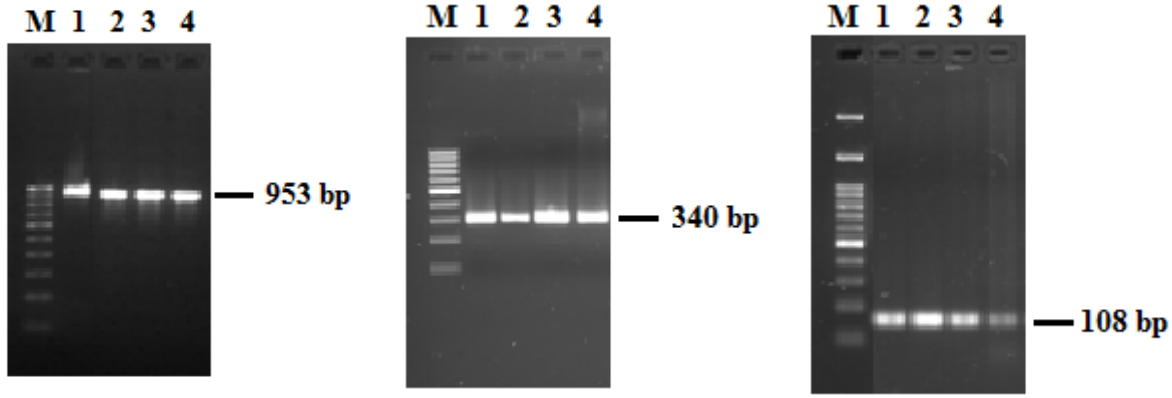
PCR amplification for *Aeromonas* sp. included the pre-denaturation at 95°C for 30 sec, subsequently followed by 25 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, elongation at 72°C for 1 min and final extension at 72°C of 8 min. Similarly, the protocol involved for *E. coli* involved the pre-denaturation at 94°C for 60 sec followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 60 sec, elongation at 72°C for 1 min and final extension at 72°C of 8 min. In addition, the PCR amplification for *Staphylococcus sp.* included the pre-denaturation at 94°C for 30 sec, 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, elongation at 72°C for 1 min and final extension at 72°C of 8 minutes. Amplicons were analyzed using 1.5% agarose gel electrophoresis.

RESULTS

Three samples from four different aquafarms were investigated to elucidate the occurrence of pathogenic bacteria such as *Aeromonas sp.*, *E. coli* and *Staphylococcus sp.* Morphological characterization of bacterial isolates revealed that about 66.67% bacterial isolates (FPA 1 to FPA-4, FPE-5 to FPE-8) were gram-negative and rod shaped (n = 8) and the rest 33.33% of the bacterial isolates (FPS-9 to FPS-12) were gram-positive cocci (n = 4). The biochemical characterization of twelve bacterial isolates showed wide variations among the bacterial isolates (Table 2).

All bacterial isolates, including reference strains ATCC 7688 (*Aeromonas hydrophila*), ATCC 25922 (*E. coli*), ATCC 25923 (*Staphylococcus aureus*) were found to be catalase positive. Four isolates (FPA-1 to FPA-4) including its reference strains were oxidase positive. Indole test showed that eight gram-negative isolates (FPA 1 to FPA-4, FPE-5 to FPE-8) were indole positive. Methyl red test showed that eight isolates (FPE-5 to FPE-8, FPS-9 to FPS-12) showed positive results and the remaining four were MR negative. The Voges-Proskauer test revealed that eight isolates (FPA-1 to FPA-4, FPS-9 to FPS-12) were VP positive. Eight isolates (FPA-1 to FPA-4, FPS-9 to FPS-12) were found to be citrate positive and four isolates (FPS-9 to FPS-12) including reference strains revealed urease positive. All bacterial isolates were found to be fermentative during oxidation fermentation test. Further, triple sugar iron, esculin and carbohydrate fermentation test demonstrated vast differences among bacterial isolates (Table 2). Except for four gram-positive bacterial isolates, all the gram-negative isolates were motile, including the reference strains (ATCC 7688 and ATCC 25922). Based on the biochemical result and Bergey's manual of bacteriology, four isolates (FPA-1 to FPA-4) were identified as *Aeromonas sp.*, four (FPE-5 to FPE-8) were identified as *E. coli* and the remaining four (FPS-1 to FPS-4) were identified as *Staphylococcus sp.* (Table 2).

Molecular identifications of bacterial isolates were performed using genus-specific primers (Table 1). The study suggested that the bandwidth of respective PCR products of isolated bacteria assured their identification such as *Aeromonas sp.* with an amplicon size of 953 bp (Figure 2a), *E. coli* with an amplicon size of 340 bp (Figure 2b) and *Staphylococcus sp.* with an amplicon size of 108 bp (Figure 2c).



(a) (b) (c)

Figure 2. PCR amplification using genus specific primers revealed (a) *Aeromonas sp.* with amplicon size of 953 bp (Lane represented FPA-1 to FPA-4); (b) *E. coli* with amplicon size of 340 bp (Lane represented FPE-5 to FPE-8); and (c) *Staphylococcus sp.* with amplicon size of 108 bp (Lane represented FPS-9 to FPS-12).

Table 2. Biochemical characterization of different bacterial pathogens isolated from different aquaculture farms such as *Aeromonas sp.* (FPA-1 to FPA-4); *E. coli* (FPE-5 to FPE-8) and *Staphylococcus sp.* (FPS-9 to FPS-12) along with their reference strains *i.e. Aeromonas hydrophila* (ATCC 7688), *E. coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923).

SI.	Bacterial isolates	Shape/size	Gram staining	Cat test	Coa test	Oxi test	Indole test	MR test	VP test	OF test	Cit test	Ure test	TSI test	ES test	CF test				Mort test	Suspected organism
															G	F	L	S		
1.	FPA-1	Rod	-	+	NP	+	+	-	+	Fer	+	-	K/A	+	+	+	+	+	+	*Aeromonas sp.*
2.	FPA-2	Rod	-	+	NP	+	+	-	+	Fer	+	-	K/A	+	+	+	+	+	+	*Aeromonas sp.*
3.	FPA-3	Rod	-	+	NP	+	+	-	+	Fer	+	-	K/A	+	+	-	+	+	+	*Aeromonas sp.*
4.	FPA-4	Rod	-	+	NP	+	+	-	+	Fer	+	-	K/A	+	+	-	+	+	+	*Aeromonas sp.*
5.	FPE-5	Rod	-	+	NP	-	+	+	-	Fer	-	-	A/A	NP	+	-	+	Facul	+	*E. coli*
6.	FPE-6	Rod	-	+	NP	-	+	+	-	Fer	-	-	A/A	NP	+	-	+	+	+	*E. coli*
7.	FPE-7	Rod	-	+	NP	-	+	+	-	Fer	-	-	A/A	NP	+	-	+	Facul	+	*E. coli*
8.	FPE-8	Rod	-	+	NP	-	+	+	-	Fer	-	-	A/A	NP	+	-	+	+	+	*E. coli*
9.	FPS-9	Cocci	+	+	+	-	-	+	+	Fer	+	+	NP	NP	+	+	+	+	-	*Staphylococcus sp.*
10.	FPS-10	Cocci	+	+	+	-	-	+	+	Fer	+	+	NP	NP	+	+	+	+	-	*Staphylococcus sp.*
11.	FPS-11	Cocci	+	+	+	-	-	+	+	Fer	+	+	NP	NP	+	+	+	+	-	*Staphylococcus sp.*
12.	FPS-12	Cocci	+	+	+	-	-	+	+	Fer	+	+	NP	NP	+	+	+	+	-	*Staphylococcus sp.*
13.	ATCC 7688 (Reference)	Rod	-	+	NP	+	+	-	+	Fer	+	-	K/A	+	+	+	+	+	+	*A. hydrophila*
14.	ATCC 25922 (Reference)	Rod	-	+	NP	-	+	+	-	Fer	-	-	A/A	NP	+	-	+	Facul	+	*E. coli*
15.	ATCC 25923 (Reference)	Cocci	+	+	+	-	-	+	+	Fer	+	+	NP	NP	+	+	+	+	-	*S. aureus*

NB: Biochemical characrization: Catalase test (Cat test); Coagulase test (Coa test), Oxidase test (Oxi test); Methyl Red test (MR test); Voges-Proskauer test (VP test); Oxidation fermentation test (OF test: Fer- Fermentative, Facul- Facultative, Oxd- Oxidative); Citrate test (Cit test); Urease test (Ure test); Triple-Sugar-Iron test (TSI test); Esculin test (ES test); Carbohydrate fermentation test (CF test); Carbohydrates: (G: Glucose, F: Fructose, L: Lactose, S: Sucrose); Mortality test (Mort test), Not Performed (NP).

DISCUSSION

Labeo rohita is being cultivated along with other major and minor carps are highly perishable, but differ significantly based on their feeding habits, environment and culture practice of farmer. Fish harbours pathogenic bacterial strains, which can be hazardous to human and animals. Majority of foodborne illness linked to fish arises by consuming uncooked or unprocessed fish and cross contamination of fish during handling that results in economic losses.

Three different bacterial strains have been isolated and identified in the present study. The isolation of opportunistic pathogen *E. coli* from *Labeo rohita* indicated faecal and environment contamination of aquaculture farm resulting from livestock manure. Several workers have sustained the occurrence of *E. coli* in the fish population [23,24,25,26,27]. Further, E. coli in aquaculture farms is attributed to poor fish handling processes [27]. Gram-positive *Staphylococcus sp.* have been isolated and identified in fish samples including *Labeo rohita* has been substantiated by several studies [26,27,28]. *Staphylococcus* sp. is reported to cause foodborne diseases by producing enterotoxins responsible for gastroenteritis after consumption [22]. Several studies have isolated and identified *Aeromonas sp.* from aquaculture farms [29]. Besides, the high prevalence of *Aeromonas sp.* in *Labeo rohita* have been reported by several workers [24,30,31]. The pathogenic *Aeromonas sp.* is associated with severe bacterial-mediated infections in freshwater aquaculture and emerging as potential threats to public health.

The detection of isolated bacterial strains like *Aeromonas sp., E. coli* and *Staphylococcus sp.* reflects environmental and faecal contamination [32]. The present study indicated that the relative distribution and occurrence of bacterial pathogens in freshwater aquafarms of Bargarh district, which might be due to the improper culture practices by farmers. Besides, the poor management practices lead to contamination with pathogenic bacterial strains such as *Staphylococcus aureus, E. coli, Salmonella typhi* and *Aeromonas hydrophila* in freshwater.

CONCLUSION

Labeo rohita is widely cultured and consumed freshwater fish in India. Pathogenic bacterial strains including *Aeromonas sp., E. coli* and *Staphylococcus sp.* are major contributors to bacterial mediated diseases in fish species. The present study highlighted the presence of bacterial pathogens in freshwater aquaculture indicating the contamination of aquatic habitat. Unhygienic handling practices of infected fish and inadequate cooking practices further contribute the transmission of bacterial pathogens, posing significant health hazards. The study suggested an urgent need for improved aquaculture practices, effective surveillance methods and a stringent quality control system. These are essential to raise awareness among fish consumers to mitigate the health risks of fish diseases caused by opportunistic bacterial pathogens.

CONFLICT OF INTEREST

None.

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