Original Research Article

Bacterial assessment and Isolation of antibiotic resistant bacteria from the Indian major carp, *Labeo rohita*

Abstract

A bacteriological study of fish is critical to protecting people from potential hazards and serving as an indicator of water contamination. The study was carried out to investigate microorganisms from the *Labeo rohita*. A total bacterial load was measured in the skin, gills, and intestine. It had a total bacterial load (TBL) of 1.54×10^9 CFU/g on average. The intestine had the highest average TBL of 2.09×10^9 CFU/g, while the gill had the lowest average TBL of 1.08×10^9 CFU/g. Isolated bacteria were grouped based on colony characteristics and then subjected to morphological, physiological, and biochemical analysis for identification up to the genus or species level. *Staphylococcus aureus* and *Vibrio* spp. were identified as the two microorganisms. After a thorough examination and PCR analysis, the two bacteria, *S. aureus* and *Vibrio spp.*, were isolated. S. aureus was detected in skin samples from R3 and R5 fish. S. aureus was isolated from R3, R4, and R5 in the gills. S. *aureus* was found in intestinal samples R2, R3, R4, and R5. The presence of *Vibrio spp.* was found in the intestines of all fish samples tested, as well as the skin of R4 and R5, and the gills of R2, R3, R4, and R5. The 16S rRNA gene was amplified in all *S. aureus* isolates, while the Tox-R gene was amplified in all *Vibrio spp* isolates. The isolated *S. aureus* and *Vibrio* tested positive for the 16s rRNA and Tox-R genes, respectively. An antibiotic sensitivity test revealed that the majority of *S. aureus* and *Vibrio spp* were susceptible to azithromycin, ciprofloxacin, and tetracycline but resistant to amoxicillin and streptomycin.

Keywords: Microbial evaluation; *Labeo rohita*; Total bacterial load; Antibiotic sensitivity

1. Introduction

Bangladesh is known as the land of rivers, which is endowed with very considerable marine, estuarine, and inland water, having great fishing potential [1]. “Fisheries sector is playing a very vital role regarding employment generation, protein supply, and foreign currency earning and poverty alleviation” [2]. “This sector is contributing 2% of the total export earning, 3.61% of national GDP and 25.71% to agricultural sector” [3]. About 2 million people are directly or indirectly involved in this sector. Aquaculture in Bangladesh have been expanded, diversified

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and technologically advanced day by day. Bangladesh is ranked third position in global aquaculture production. Aquaculture also opens up employment opportunities within the rural economy. The demand for fish in Bangladesh has been increasing rapidly, due to both population growth and gradual economic growth. Aquaculture is an emerging industrial sector which requires continued research with scientific and technical developments and innovations [4]. "Fish is one of the important sources of quality animal proteins and availability and affordability is better for fish in comparison to other animal protein sources" (Sumon et al. 2022a). "Fish serves as a health-food for the affluent world owing to the fish oils which are rich in polyunsaturated fatty acids (PUFAs), especially ω-3 PUFAs and at the same time, it is a health-food for the people in the other extreme of the nutritional scale owing to its proteins, oils, vitamins and minerals" [6]. "*L. rohita (*rohu/Rui) is the most important commercial fish species of South Asia including Bangladesh with very high market price and demand" [7].

Microorganisms are widely distributed in nature wherever favorable environmental factors are present for their growth [8]. Usually food supply, temperature and moisture are the main factors that determine the growth of microorganisms. They derive their energy from host organisms. Bacteria are one of the most important microorganisms present in fishes and are responsible for economic loss due to mortality and contamination of fish food [9]. Bacteria are found on all the outer surfaces (gill and skin) and intestine of fishes [10]. Spoilage and pathogenic bacteria are important for food safety as well as for human health [11]. "Bacteria are also known to cause zoonotic disease to the handlers and final consumers" [12]. "Human infection results from contamination of hands and utensils during processing operations especially at evisceration" [13]. "Fishes are also known to transmit human food borne infection and intoxication caused by *Salmonella spp., Staphylococcus spp., Vibrio spp.* and *Aeromonas spp"* [14,15].

"Fishes are in association with microbes present in the aquatic environment, and they receive bacteria from the aquatic environment through water and food. Being rich in nutrients, fish kidney, liver, gill, spleen and intestine confers a favorable niche for microorganisms, which in turn is generally colonized by large number of heterotrophic bacteria, and also there is a complex microbial ecosystem" [16]. Kidney, liver, gill, spleen and intestine microbes of fish have been studied by many researchers [17]. The microbial community has an important role in the health and nutrition of the host (Sumon et al., 2022b). "The indigenous microflora of the fish digestive tract has been traditionally investigated by conventional culture-dependent

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methods including cultivation on selective or non-selective media followed by isolation and phenotypic characterization" [19]. "Traditionally, studies on fish-associated microorganism's involved culture-dependent techniques of dubious sensitivity, which highlighted only the bacteria typically the aerobic heterotrophic bacterial component" (Austin 2006; Sumon et al. 2022c). Traditional culture methods are increasingly being replaced by molecular approaches [22]. The routine detection of bacterial strains can be accomplished with success using PCR techniques, which are quick and reliable [23]. Therefore, the current study intends to evaluate the bacteriological examination of a commercially important fish species, *L. rohita*, using biochemical tests, molecular characterization via PCR, and antibiotic sensitivity of isolated bacteria.

2. Materials and Methods

2.1 Sample collection and preservation

The present study was conducted during the period from February 2019 to August 2019. Sample collection site was The Brahmaputra River, Jamalpur sadar Upazila (Figure1). In total five specimens of *L. rohita* were collected directly from the fishermen at the spot just after their fishing. The fish samples were packed individually in separate polythene bags. Fish in bags were preserved in an ice box with crushed ice around the fish samples (Figure 2). The whole ice box was filled completely with the crushed ice (Figure 2). Such preserved fish samples were immediately taken to the Microbiology laboratory, Department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh for bacteriological investigation. Specimens were dissected with a sterile knife and different organs were taken out and preserved by refrigeration at 4°C for further analysis.

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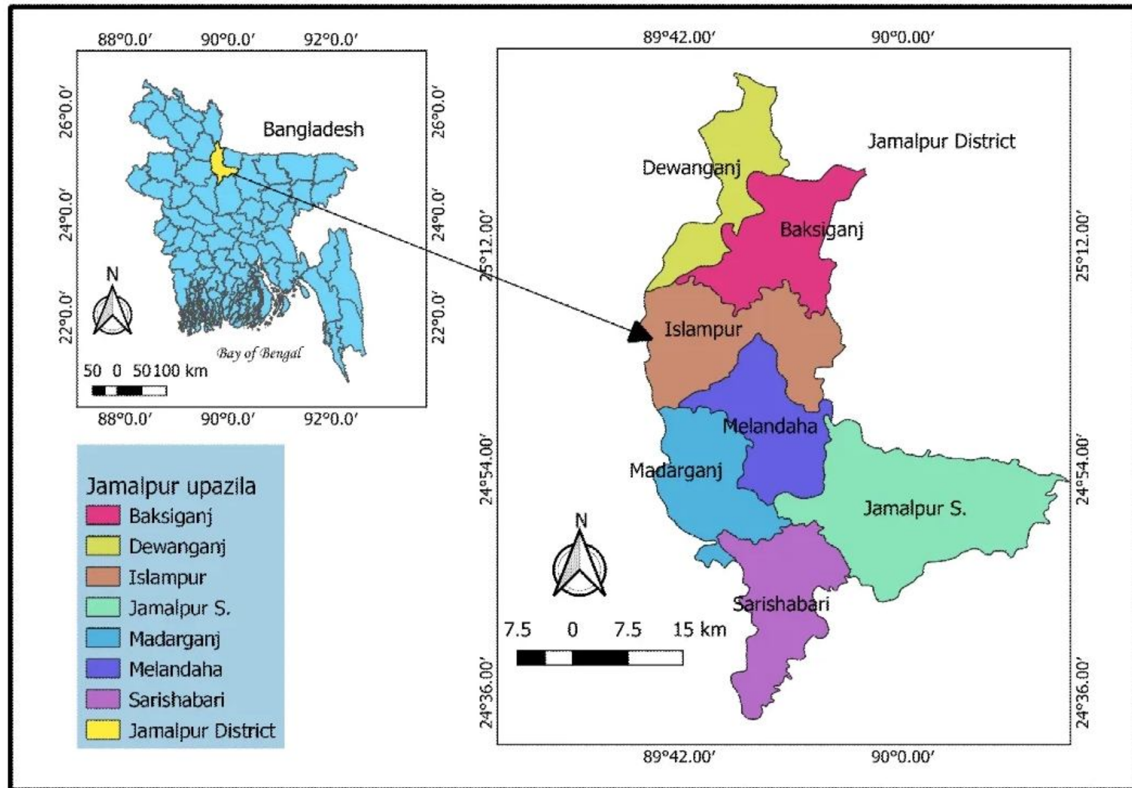

Figure 1. The map showing the study area of the Brahmaputra River, Bangladesh

Figure 2. Collection and preservation of fresh samples direct from the fishing spot

2.2. Bacteriological analysis

2.2.1. Bacterial load determination

"For the determination of total bacterial load, 0.1 ml of each ten-fold dilution was transferred and spread onto duplicate Plate count agar (PCA) using a fresh pipette for each dilution. The diluted samples were spread as quickly as possible on the surface of the plate with a sterile glass spreader. One sterile spreader was used for each plate. The plates were then kept in an incubator at 37°C for 24-48 hours. Following incubation, plates exhibiting 30-300 colonies were counted. The average number of colonies in a particular dilution was multiplied by the dilution factor to obtain the total bacterial load. The results of the total bacterial load were expressed as the number of organism or colony forming units per gram (CFU/g) of fish sample. Colony characteristics such as shape, size, surface texture, edge and elevation, color and opacity developed after 24 hours of incubation at 37°C were recorded" [24].

2.2.2. Identification and Characterization of Bacteria

"The isolated bacterial colonies were identified on the basis of their morphological, physiological and biochemical characters. These culture were subjected to various biochemical tests such as gram staining, motility, catalase, indole, methyl red (MR), voges Proskauer (VP), and sugar fermentation tests for identification of phosphate solubilizing bacteria using Bergey's manual of systematic bacteriology" [25]. The bacteria such as *vibrio spp* and *S. aureus* were isolated from the various parts of fish samples.

2.2.3. Biochemical Confirmation

"Five typical colonies were subcultured on nutritional agar (Oxoid, England) for biochemical confirmation by indole production from each Rainbow agar O157 plate. The agar plates were incubated for 18-24 hours at 37°C. One colony from the pure culture on nutrient agar was inoculated into a tube of Oxoid (England) tryptone/tryptophan medium and cultured at 37°C for 24 hours. The tube was then filled with 1 ml of Kovach's (Oxoid, England) and allowed to stand at room temperature for 10 minutes. The appearance of red suggests a positive reaction" [26].

2.3. Molecular characterization by PCR

2.3.1. Molecular detection of Staphylococcus aureus

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“The DNA template was prepared by boiling method. In brief, for the preparation of DNA template, a single colony of bacteriological culture of *S. aureus* was taken in 250 µl of DW in Eppendorf tube, mixed well and then boiled for 10 minutes. After boiling the tubes were immediately placed on ice for cold shock followed by centrifugation at 10,000 rpm for 10 minutes at 4°C. The supernatant was collected which was further used as DNA template” [27]. PCR reaction was performed to detect *S. aureus* specific 16S rRNA gene from isolated *S. aureus* in a thermal cycler (Astec, Japan). Primers used for this purpose is presented in table 1. Each 20 µl reaction mixture was consisted of 3 µl genomic DNA, 10 µl PCR master mixture (Promega, USA) and 1 µl of each of the two primers with the final volume adjusted to 20 µl with 5 µl of nuclease free water. Amplification was done by initial denaturation at 95°C for 5 minutes, followed by denaturation at 94°C for 1 minute. Annealing temperature of primers was 53°C for 1 minute with an increment of 72°C for 1 minute. The final extension was conducted at 72°C for 7 minutes. The total reaction was performed at 35 cycles. The amplified PCR products were resolved by electrophoresis in 2% agarose gel at 80v for 30 minutes, stained with ethidium bromide and finally visualized under UV trans-illuminator.

Table 1. Primers used in PCR for *S. aureus*

Primer	Sequence (5'-3')	Target	Amplicon size(bp)	Reference
SauF234	CGATTCCUTAGTAGCGGCG	*16S rRNA* Gene	1267	[28]
SauR150	CCAATCGCACGCTTCGCC			

2.3.2. Molecular detection of Vibrio by PCR

A pure bacterial colony of *Vibrio* was mixed with 100 µl of distilled water which were boiled for 10 minutes and then immediately kept on ice for ice shock. Finally, centrifugation was done at 10000 rpm for 10 minutes. The supernatants were collected and used as DNA template for PCR [27]. *Vibrio* genus specific PCR was performed to amplify *ToxR* gene using previously published primers (Kim et al., 1999). The list of primers is shown in Table 2.

Table 2. Primers used in PCR for *Vibrio spp*

Primer	Sequences (5’-3’)	Target	Amplicon Size (bp)	References

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Tdh-F	GGTCTAAATGGCTGACATC	*toxR* gene	689	(Kim et al., 1999)
Tdh-R	CCACTACCACTCTCATATGC			

2.4. Antibiotic sensitivity test

All isolates of *Staphylococcus aureus* and *Vibrio spp* were tested for their antibiotic sensitivity *against five commonly used antibiotic agents by disc diffusion method according to the guidelines* of Clinical and Laboratory Standard Institute, CLSI [30]. Colonies were selected from PCA agar plate. The top of the colony was touched with a loop and was transferred into nutrient broth. The broth was streaked onto Mueller Hinton agar plate by using sterile glass spreader homogenously. The antibiotic discs were placed individually using sterile forceps and then gently pressed down onto the agar. The plates were kept inverted and incubated at 37°C for overnight. After incubation the diameter of the inhibitory zone (including diameter of the discs) was measured in millimeters with a scale. Commercially available antibiotic discs (Himedia, India) were used to determine the antibiotic sensitivity patterns. The antibiotic agents that were used against the isolated bacterial species are presented in the Table 3.

Table 3. Antibiotic agents with their disc concentration

Name of the antibiotic agents	Symbol	Disc concentration (µg/disc)
Ciprofloxacin	CIP	05
Azithromycin	AZM	30
Amoxycillin	AMX	30
Streptomycin	S	10
Tetracycline	TE	30

2.5. Recording and interpreting results

The zones of growth inhibition were compared with the zone size interpretative tables (Tables 4 and 5) provided by CLSI (Weinstein et al., 2020). Antibiotic sensitivity testing results were recorded as sensitive, intermediately sensitive, and resistant.

Table 4. Zone diameter interpretative standards for *Vibrio spp*

Antibiotic agents	Zone diameter (millimeter)		
	Resistant	Intermediate sensitive	Sensitive
Amoxycillin	≤ 13	14-17	≥ 18

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Azithromycin	≤ 13	14-17	≥ 18
Ciprofloxacin	≤ 20	21-30	≥ 31
Streptomycin	≤ 12	12-14	≥ 15
Tetracycline	≤ 11	12-14	≥ 15

Table 5. Zone diameter interpretative standards for *S. aureus*

Antibiotic agents	Zone diameter standard in mm		
	Resistant	Intermediate sensitive	Sensitive
Amoxycillin	≤ 19	-	≥ 20
Azithromycin	≤ 13	14-22	≥ 23
Ciprofloxacin	≤ 15	16-20	≥ 21
Streptomycin	≤ 12	-	≥ 13
Tetracycline	≤ 15	16-25	≥ 26

3. Results

3.1 Total Bacterial Load (TBL)

The result of the total bacterial load in Rui is given in Table 6. The average TBL in Rui was 1.54×10^9 CFU/g. Among the five Rui (R1, R2, R3, R4, R5) the average bacterial load in their skin, gill and intestine were 1.46×10^9 CFU/g, 1.08×10^9 CFU/g and 2.09×10^9 CFU/g respectively. The maximum number of bacterial count was 3×10^9 CFU/g in the intestine of R1 while minimum number was found in the gill of R3, and it was 0.08×10^9 CFU/g. The highest number of bacterial load in skin was found 2.5×10^9 CFU/g in R1 while the lowest number of bacterial load in skin was found 0.75×10^9 CFU/g in R3. The highest number of bacterial load was found in gill 2.5×10^9 CFU/g in R5 and the lowest number of bacterial load was found in gill 0.08×10^9 CFU/g in R3.

Table. 6. Total bacterial load in three different organs of five *L. rohita* specimens investigated

Total Bacterial Load CFU/g of different organs in each sample								
Fish		**R1**	**R2**	**R3**	**R4**	**R5**	Average in Organ	Average in Fish
Organs	Skin	2.5×10^9	1.2×10^9	0.75×10^9	0.88×10^9	2×10^9	1.46×10^9	1.54×10^9
	Gill	0.65×10^9	1.5×10^9	0.08×10^9	0.7×10^9	2.5×10^9	1.08×10^9	
	Intestine	3×10^9	1.85×10^9	2.6×10^9	1.36×10^9	1.68×10^9	2.09×10^9	

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3.2 Confirmation of S. aureus and Vibrio spp in three different organs of five Specimens of Labeo rohita

The isolated Staphylococcus aureus and Vibrio spp from different organs of the investigated Rui fish presented in Table 7. Skin samples from R3 and R5 were found to contain S. aureus. In the gills, S. aureus was separated from R3, R4, and R5. Intestinal samples R2, R3, R4, and R5 carried S. aureus. There was evidence of the presence of Vibrio spp in the intestines of all of the samples of Rui that were evaluated, as well as in the skin of R4 and R5, and the gills of R2, R3, R4 and R5.

Table 7. Presence of Staphylococcus aureus and Vibrio spp in three different organs of five specimens of *L. rohita*.

Labeo rohita	***S. aureus***					***Vibrio spp***				
Specimen no Organ	R1	R2	R3	R4	R5	R1	R2	R3	R4	R5
Skin	-	-	+	-	+	-	-	-	+	+
Gill	-	-	+	+	+	-	+	+	+	+
Intestine	-	+	+	+	+	+	+	+	+	+

'+' and '–'signs reveal the present and absent results respectively.

3.3. Cultural, morphological and staining Characterization

The characteristics of the isolates are shown in Table 8-11. S. aureus have developed yellowish color colonies on mannitol salt agar as well as positive in catalase test. Gram positive, cocci, arranged in grapes like cluster. S. aureus shown grapes like cluster under light Microscope. In carbohydrate fermentation test of S. aureus showed production of acid only. In biochemical test, S. aureus found positive for MR and indole test whereas, negative for VP test.

On thiosulfate-citrate-bile salts sucrose (TCBS) agar, Vibrio spp. developed colonies that were yellow or green in color. Gram-negative, Vibrio comma-shaped bacteria were observed under a light microscope. Vibrio spp found catalase positive as well as positive on destrose, maltose, sucrose and mannose in the fermentation test. Vibrio spp. was also identified using the MR, VP, and Indole assays.

Table 8. Cultural and Gram stanning characteristics.

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Numbers of Isolates	Agar media	Gram Staining Properties of Isolates	Remarks
15	MS	Gram (+) ve, cluster form, crystal violet color	*Staphylococcus* like
10	TCBS	Gram (-) ve, large rod-shaped bacteria	*Vibrio* like
15	PCA	Gram (+) ve, cluster form, crystal violet color	*Staphylococcus* like
15	PCA	Gram (-) ve, large rod-shaped bacteria	*Vibrio* like

'+' and '–' signs reveal the positive and negative results respectively.

Table 9. Biochemical characteristics of the tested isolates

Characters		***Staphylococcus aureus***	***Vibrio spp***
Gram stain		+	-
Motility		-	+
Shape		Grape-like clusters, cocci shaped	Large rod shaped
Oxidase		-	+
Catalase		+	+
Coagulase		+	+
OF Test		Fermentative	Fermentative
Nitrate reduction		+	+
Citrate utilization		+	+
Acid and gas production from sugar	Dextrose	+	+
	Sucrose	+	+
	Maltose	+	+

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	Lactose	+	+
	Manitol	+	+
Decarboxylase test	L-Arginine	+	-
	L-Lysine	+	+
	L-Ornithine	-	+
Methyl red test		+	-
Voges-proskauer		+	-
Indole production		-	+
H_2S production		-	-
Gelatin lequifaction		+	+
Growth at	4°C	-	-
	37°C	+	+
	40°C	-	-
Growth in NaCl solution	0%	+	+
	1%	+	+
	2%	+	+
	3.5%	-	-
	4%	-	-

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3.4. Molecular analysis

3.4.1. Confirmation of *S. aureus*

The optimized PCR assay was able to successfully amplify the target 16S rRNA gene (1267 bp fragment) from the DNA templates of all isolated S. aureus. Result of PCR for S. aureus is shown in Fig. 3.

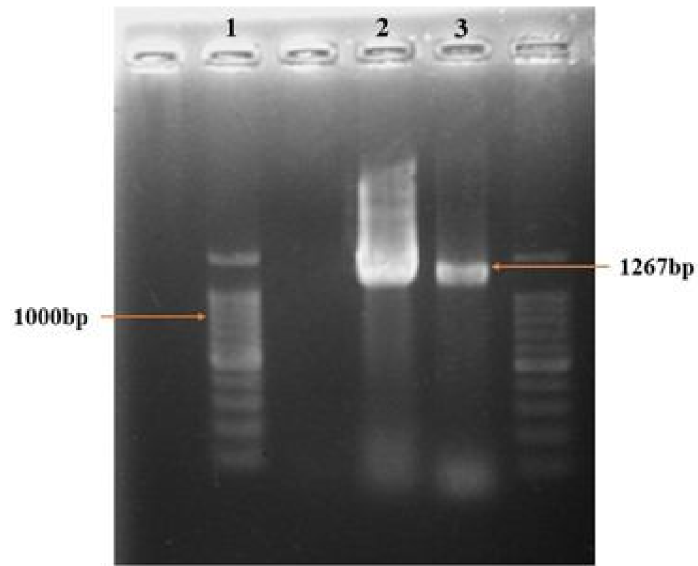

Figure 3. The 16SrRNA gene-based PCR of S. aureus. Lane 1: 1000 bp ladder; Lane 2, and 3: tested samples were positive for 16S rRNA gene.

3.4.2. Confirmation of *Vibrio*

PCR primers targeting tox-R gene of Vibrio amplified 689 bp fragments of DNA confirmed the identity of Vibrio. Result of PCR for Vibrio is shown in Fig. 4.

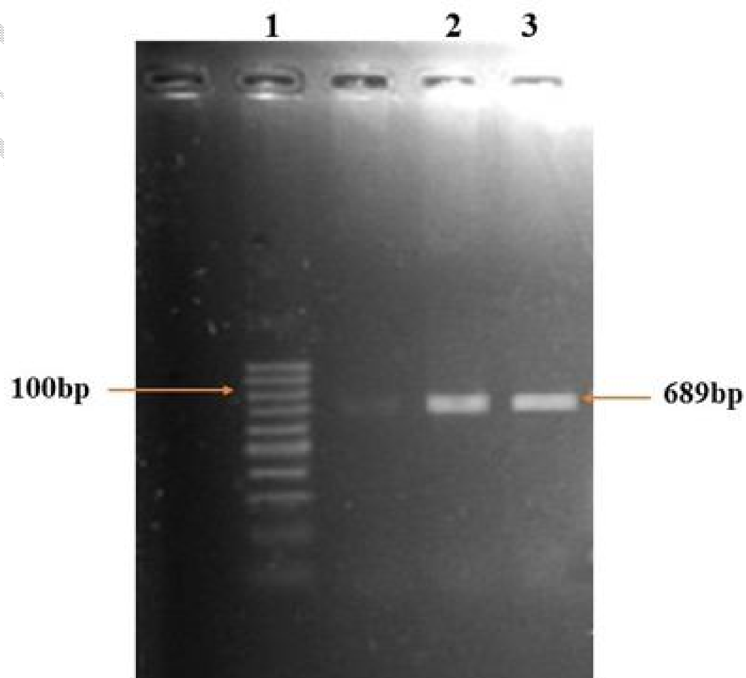

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Figure 4. Tox-R gene-based PCR for *Vibrio*. Lane 1: 100 bp DNA ladder, Lane 2, 3: tested sample was positive for tox-R gene.

3.5. Antibiogram profile

3.5.1 Antibiotic sensitivity of isolated S. aureus

The overall sensitivity pattern of S. aureus isolates is presented in Figure 5. All isolates were found to be 100% resistant to amoxicillin, and streptomycin. 100% and 93.33% were sensitive to ciprofloxacin and azithromycin. Furthermore 6.67% were intermediate sensitive to tetracycline (Table 10).

Table 10. Results of antibiotic sensitivity of isolated *S. aureus*

Name of bacteria	**Antibiotic agents**	**Antibiogram profile**		
		Sensitive (%)	Intermediate (%)	Resistant (%)
S. aureus	Amoxicillin	0	0	100
	Azithromycin	93.33	6.67	0
	Ciprofloxacin	100	0	0
	Streptomycin	0	0	100
	Tetracycline	0	6.67	0

3.5.2. Antibiotic sensitivity of isolated *Vibrio spp*

The overall sensitivity pattern of *Vibrio spp* isolates are presented in Figure 5. The results showed that 100%, 93.33%, and 6.67% were sensitive to tetracycline, azithromycin, and ciprofloxacin respectively. However, 93.33% and 6.67% were intermediate sensitive to azithromycin and ciprofloxacin. The bacteria were 100% resistant against amoxicillin and streptomycin (Table 11).

Table 11. Results of antibiotic sensitivity of isolated *Vibrio spp*

Name of bacteria	**Antibiotic agents**	**Antibiogram profile**		
		Sensitive (%)	Intermediate (%)	Resistant (%)
Vibrio spp	Amoxicillin	0	0	100
	Azithromycin	93.33	6.67	0
	Ciprofloxacin	6.67	93.33	0
	Streptomycin	0	0	100

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Tetracycline	100	0	0

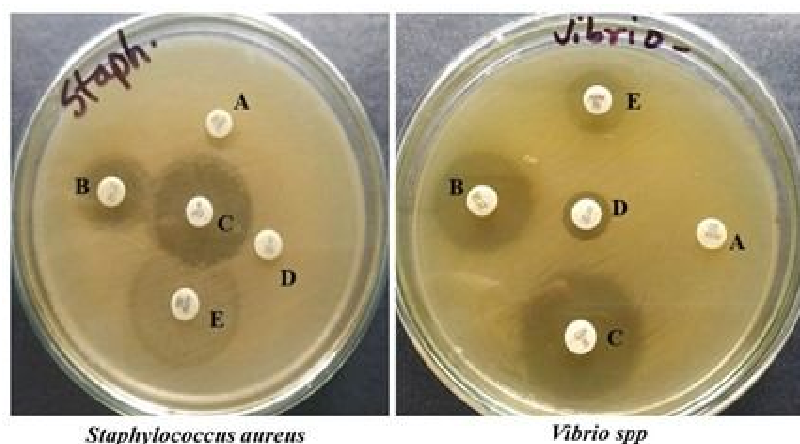

Figure 5. Antibiotic sensitivity for Staphylococcus aureus and Vibrio spp by disc diffusion method. A. Amoxycillin, B. Tetracycline, C. Ciprofloxacin, D. Streptomycin, E. Azithromycin

4. Discussion

Due to variations in species, habitats, and feeding habits, fish are extremely perishable and subject to wide quality variations. They can also act as carriers of a variety of microbial and other health risks. Fresh and frozen fish should be free of *Vibrio*, according to a recommendation from the International Association of Microbiological Societies [31]. Sarkar et al., (2020) reported that fish from local markets were more contaminated with *Vibrio spp.* than super shops in capital of Bangladesh which is the third freshwater fish producing country in the world. Contamination may be caused by foodborne pathogens which are naturally present in aquatic environments, such as *Vibrio spp.*. The most significant factor contributing to fish mortality may be bacterial infestation [33]. The present study was isolated two important bacteria, *S. aureus* and *Vibrio spp.*.

According to the findings of the bacterial assessment study, the average total bacterial load in *Labeo rohita* was 1.54×10^9 CFU/g. Shamsuzzaman et al., (2011) stated that the total bacterial load in *L. rohita* ranged from 2.94×10^5 CFU/g to 1.827×10^6 CFU/g. As per organ of Rui fish, the average bacterial load in the skin, gill, and intestine of the five Rui (R1, R2, R3, R4, R5) were 1.46×10^9 CFU/g, 1.08×10^9 CFU/g and 2.09×10^9 CFU/g respectively.

In this study, two different types of bacteria were isolated from *L. rohita*. The isolated bacteria were *S. aureus* and *Vibrio spp*. The results of isolation are similar with the findings of

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Adebayo-Tayo et al., (2012); Stratev et al., (2021). The bacterial isolates obtained from the intestine of fish are *Salmonella* spp., *Proteus* spp., *Staphylococcus* spp., *Pseudomonas* spp., *Escherichia coli*, *Shigella* spp, and *Vibrio* spp. *Staphylococcus* spp. isolates were gram positive, non-motile, oxidase-negative, catalase positive, coagulase positive, oxidative fermentative, nitrate positive, citrate positive, methyl red positive, vogas-proskauer positive, indole negative, H_2S negative which is similar to present findings [36].

PCR based molecular detection of these pathogens in the raw fish products could be remarkably contributing to clarify the actual role in *Staphylococcal* food poisoning and other clinical symptoms associated with the consumption of raw fish products [37]. PCR could detect more specifically *S. aureus* than other method [38]. In present study, the 16SrRNA gene-based PCR for *S. aureus* and *Tox-R* gene-based PCR for *Vibrio* showed that the isolated *S. aureus* and *Vibrio* were positive to *16s rRNA* and *Tox-R* gene, respectively. Detection of the virulence genes in *V. parahaemolyticus* is only for determination of pathogenic strains whereas *toxR* genes can be used to determine all isolates either pathogenic or non-pathogenic and also functions as regulator for expression of the virulence factor genes in *V. parahaemolyticus* [39]. This indicates that the *Tox-R* sequence determination has a greater potential for the identification of genetically close bacterium genera, such as the genus *vibrio*, than does conventional biochemical assays.

Antibiotic sensitivity test is an important task that was performed to show Antibiotic sensitivity pattern of significant bacterial isolates [40]. Results of antimicrobial susceptibility test showed that most of the isolates of *S. aureus* were sensitive to azithromycin, tetracycline and ciprofloxacin but resistant to amoxicillin and streptomycin. On the other hand, most of the isolates of *Vibrio spp* were sensitive to azithromycin, tetracycline and ciprofloxacin but resistant to amoxicillin and Streptomycin, which was closely related with the findings of Memon et al., (2013).

5. Conclusions

Considering the results presented in the microbial assessment of *L. rohita* as the bacterial flora in fish depends on the environment, the total bacterial load, and the prevalence of bacterial flora of fishes might vary from fish species and organs. Freshwater microflora adapted in Rui body was identified. For these purpose, total bacterial load of Rui was understood. The bacteria were undergone their morphological, physiological, and biochemical characteristics for

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identification up to genus or Species level. These would be background information for addressing the future threat to culture condition.

Ethical Approval

The experiments were approved by Animal Ethics Committee of Bangladesh Agricultural University, and performed according to the Animal Ethics Procedures and Guidelines of the People's Republic of Bangladesh.

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