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Research article

Identification and virulence gene characterization of pathogenic bacteria from diseased *Labeo rohita* (Hamilton, 1822): Insight into aquatic animal health management in Indian aquaculture

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Abstract: Aquaculture is one of the major economic activities in India, providing livelihoods and nutritional security to millions of people. In recent times, fish diseases have come to the limelight resulting in significant economic losses. We aimed to identify pathogenicity and virulence profiling of thirty-six pathogenic bacterial strains isolated from diseased *Labeo rohita* in the district of Hooghly, West Bengal, India. The bacterial strains were characterized through a comprehensive approach involving the examination of morphological features, biochemical properties, amplification, and sequencing of the 16S rRNA, species-specific genes, and virulence genes. Considering the prevalence frequency, virulence potential, and statistical significance *Aeromonas hydrophila* and *Pseudomonas aeruginosa* were selected for a survival assay followed by the examination of histopathological features to elucidate their effects. The identified bacterial isolates were arranged based on their predominance frequency, i.e., *Aeromonas hydrophila* (25%), *Aeromonas veronii* (22%), *Pseudomonas aeruginosa* (22%), *Enterococcus faecalis* (14%), *Klebsiella pneumoniae* (6%), *Staphylococcus aureus* (6%) and *Escherichia coli* (5%). Sixteen virulence-associated genes related to

pathogenicity were amplified across the thirty-six isolates; *aer, alt, lip* and *hlyA* for *A. hydrophila*; *exoS, lasB*, *toxA, oprL* and *phzM* for *P. aeruginosa*; *entB, fimH* and *uge* in *K. pneumoniae*; *aer* in *A. veronii*; *hlyA* in *E. coli*; *hlb* in *S. aureus* and *gelE* for *E. faecalis*. The log-probit analysis revealed that *A. hydrophila* was notably more pathogenic than *P. aeruginosa*, as indicated by its lower lethal dose of 1.5×10^4 CFU/mL. Additionally, histological examination revealed notable pathological changes, including tissue degeneration, inflammatory cell infiltration and vacuolation observed in the liver, kidney, gill and intestine of the challenged fish. We highlighted several potent aquatic microbial pathogens in order to manage and prevent such aquacultural maladies.

Keywords: Rohu (*Labeo rohita*), Virulence Gene, Pathogenicity, Lethal dose, Histopathological study

1. Introduction

The population explosion on our planet will reach 9 billion by 2050, with India currently leading the way, and this soaring population faces increasing poverty and malnutrition (home to > 83 million poor). Aquaculture could be crucial for food and nutritional security with high-quality animal protein (>15%), providing livelihood and income source generation to millions of people [1]. Fishes are efficient converters of feed into protein and are proven to produce a lower carbon footprint per kilogram of output compared to other terrestrial livestock [2]. Fish proteins are cheap and easily digestible. It leads to a persistent demand in both national and international markets. India is the third largest fish-producing country in the world. During the financial year (FY) 2022–2023, the country produced approximately 1.592×10^7 metric tons of fish [3]. India is also regarded as a carp-producing country, contributing about 80% of total fish production. According to the National Fisheries Development Board (NFDB) of India, the fisheries and aquaculture sector provides employment opportunities to over 14 million people. This has generated Rupees 63,969.14 crore (≈USD 7.64×10^9) in agricultural export earnings annually [3]. However, shifting from traditional to commercial aquaculture practices has led to significant changes in fish production, generating substantial revenue through exports. Nevertheless, this shift towards intensive aquaculture practices, characterized by high stocking densities and heavy inputs of feed, fertilizers, and chemicals, can alter water quality parameters and create conditions favorable for disease-causing pathogens. The development of a particular disease is influenced by a triangular relationship between the host, the pathogens involved and the surrounding environmental conditions.

West Bengal, an eastern state of India, is a significant contributor to freshwater fish production. It boasts of producing the highest output of fish seed in the nation. The 2022–2023 report is evidence of a notable surge in fishery production in this state, with 35,290 million of fish seed and 1.684×10^6 metric tons of inland fish, as cited in the Fisheries 2023 report [3]. However, the prevalence of diseases poses a significant threat to fish farming in this region. Bacterial diseases are very common among fish and several factors like poor water quality, nutritional deficiency and high stocking density are responsible for creating a conducive environment of bacterial infection in fish. Some common bacterial diseases associated with fish are motile Aeromonas Septicemia, Edwardsiella Septicemia, Pseudomonas Septicemia, Enteric Red Mouth Disease, Columnaris Disease, Vibriosis etc. [4]. Bacterial diseases are frequently found in eggs, fries and fingerlings of fish causing high mortality [3].

A dearth of comprehensive research has focused on surveilling fish pathogenic bacteria from lakes and ponds within the Hooghly district of West Bengal, India. Hooghly district contributes 6.18% to the overall fish production of West Bengal, serving as a direct or indirect livelihood source for 25,232 fisherman families as per Directorate of Fisheries Government of West Bengal (2020–2021) [3].

Hence, this study aimed to identify and characterize the microbial agent responsible for the significant mortality in Rohu (*Labeo rohita*) in the Hooghly District of West Bengal, India, from March 2021 to February 2022. Additionally, it emphasized the importance of implementing optimal management practices in aquaculture. The study also assessed the pathogenic potential of these bacteria by examining of virulence-associated genes and histopathological analyses.

2. Materials and methods

2.1. Clinical observation and sampling

During the study period from March 2021 to February 2022, 171 farmed *L. rohita* exhibiting clinical signs were collected from 43 different sites in Hooghly, West Bengal, India (Figure 1 and Table S1). In the pre-summer and post-summer season, mass mortality was observed in Indian Major Carp (IMC). Moribund fishes with clinical observation, including unnatural swimming behavior at the surface water, multifocal dermal lesions, diffuse reddening and hemorrhage over the body surface and particularly at the base of fins, and profuse mucus secretion (Figure S1) were collected from sampling sites and brought to the laboratory under the ice-cold condition in sterile plastic bags. After surface sterilization, the kidney, muscle and liver samples of the fishes were taken out aseptically and transferred immediately into a falcon tube containing Tryptic Soy Broth (TSB, Hi-media, India) and kept in the incubator for 24 h at 37 °C. A small part of all the tissue *viz*., muscle, kidney and liver was preserved in 10% neutral buffer formalin (NBF) for histopathological study.

2.2. Screening of bacteria by differential media

Overnight bacterial culture was serially diluted up to 1×10^{-6}, spread on Tryptic Soy Agar (TSA) plate and incubated at 37 °C for 24 h to obtain a pure colony. Preliminary screening was carried out based on differences in the colony morphology, color and elevation etc. of the bacterial isolates. Single bacterial colony was picked up and transferred to different selective media (Figure S2) *viz.,* Aeromonas Isolation HiVeg Medium Base added with Aeromonas Selective Supplement (FD039) for *Aeromonas sp*. (MV884, Hi-media, India), Eosin methylene blue agar (EMB) for *Escherichia coli* (M317, Hi-media, India), Pseudomonas agar base for *Pseudomonas sp.* (M085, Hi-media, India), Thiosulfate citrate bile salts sucrose Agar (TCBS) for *Vibrio sp.* (M189, Hi-media, India), HiCrome Staph Selective Agar for *Staphylococcus sp*. (M1931, Hi-media, India), Klebsiella Selective Agar Base for *Klebsiella sp.* (M1573, Hi-media, India), and MacConkey agar for Gram-negative lactose fermenting and lactose non-fermenting bacteria isolation (M008S, Hi-media, India). After serodiagnosis, single colony was sub-cultured in Tryptic Soya Broth (TSB) and preserved in glycerol stock at −20 °C for future use.

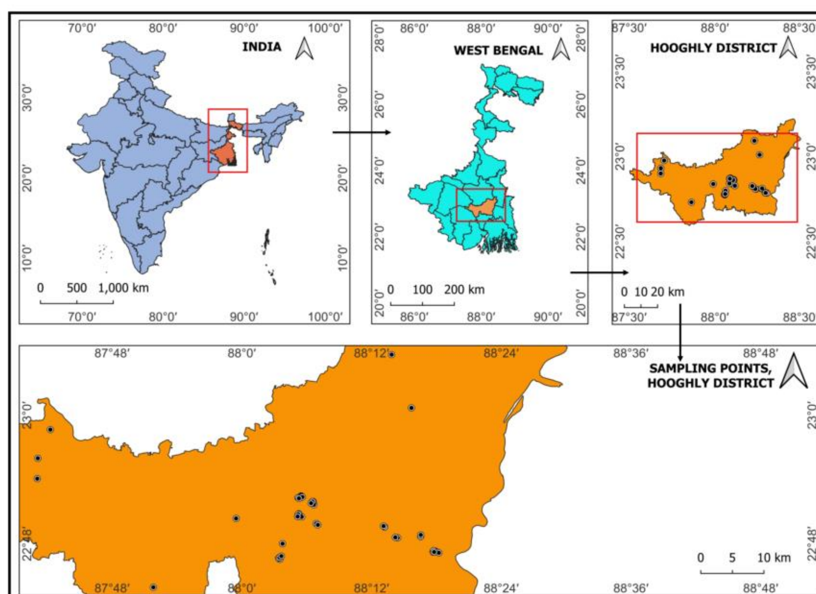

Figure 1. Map illustrates sampling sites (black dot with white circles) across Hooghly District, West Bengal, India.

2.3. Solid-phase hemolysis

The pure isolates were subjected to solid-phase hemolysis assay by streaking on a blood agar plate (Blood Agar Base) (Hi-media, India) supplemented with 2% human red blood cells [5]. The plates were incubated for 24 h at 37 °C. The results were further classified as β- hemolysis (indicated by the appearance of a clear colorless zone encircling the bacterial colony) and α- hemolysis (indicated by an incomplete transparent region) (Figure S3) [6], underscoring the significance of our research.

2.4. Biochemical characterization

The Bacterial isolates were screened by routine Gram staining, a process that strictly adhered to the manufacturer's protocol (K001, Hi-media, India). The isolates were then grouped and characterized according to colony morphology, including shape, size, edges, elevation, color and pigmentation. This was followed by a series of biochemical tests, each conducted in strict accordance with the manufacturer's instructions (KB003, Hi-media, India), including ONPG (β-galactosidase), Lysine utilization, Ornithine utilization, Urease, Phenylalanine deamination, Nitrate reduction, H2S production, Citrate utilization, Voges-Proskauer's, Methyl red, Indole, Malonate utilization, Esculin hydrolysis, Arabinose, Xylose, Adonitol, Rhamnose, Cellobiose, Melibiose, Saccharose, Raffinose, Trehalose, Glucose, Lactose, Oxidase test, Catalase test and Urease test.

2.5. Molecular identification and phylogenetic analysis

Bacterial genomic DNA was extracted by the Sarkosyl method [7]. Extracted DNA was then quantitatively measured by Eppendorf BioSpectrometer (Eppendorf, Germany) at 260 nm. The

quality of DNA was checked using 1% agarose gel containing ethidium bromide. 16S rRNA gene was amplified using the following forward primer UFF2 5′GTTGATCATGGCTCAG3′ and reverse primer URF2 5′GGTTCACTTGTTACGACTT3′ [8]. PCR thermal profile was set with an initial denaturation for 2 min at 95 °C followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 60 s and extension at 72 °C for 45 s with a final extension for 3 min at 72 °C. The PCR products were visualized under UV radiation on a 1.8% agarose gel (Figure S4), ensuring the accuracy of the amplification. Following PCR analysis with 16S rRNA primers, seven housekeeping genes have been targeted for accurate species identification. Table 1 lists the primers for housekeeping genes. After PCR analysis, 1.8% agarose gel electrophoresis was used to check for positive amplification of the targeted genes under UV. The PCR products were sequenced in forward and reverse directions using an ABI 3730xl capillary sequencer (Applied Biosystems, Foster City, CA). The forward and reverse sequences were aligned using the DNA Baser Assembler v0.7.0. version. Sequences were trimmed from both ends and BLAST-N search (http://www.ncbi.nlm.nih.gov/BLAST) was conducted with 16S rRNA sequences and housekeeping gene sequences for similar sequences. Phylogenetic analysis of 16S rRNA sequences and housekeeping gene sequences was inferred through the NJ (Neighbor-Joining) method [9] using CLC Genomics Workbench 21 [10] and MEGA 11 software respectively. The evolutionary distances were computed using NJ method and are in the units of the number of base substitutions per site [11]. To visualize the bootstrap consensus tree, 1000 replicates were taken and the associated taxa clustered together with highest bootstrap value are shown next to the branches [12].

Table 1. Primer list of housekeeping gene used to identify bacterial isolates.

Target gene	Bacterial isolate	Primer sequences (5′–3′)	Annealing temperature (°C)	Amplicon size (bp)	Ref.
gyrB	*A. hydrophila*	GGGGTCTACTGCTTCACCAA CTTGTCCGGGTTGTACTCGT	59	669	[13]
rpoB	*A. veronii*	CGTGCCGGCTTTGAAGTC ATCACGTACTTGCCTTCTTCAATA	57	224	[14]
recA	*E. coli*	CGCATTCGCTTTACCCTGACC TCGTCGAAATCTACGGACCGGA	58	780	[15]
rpoB	*K. pneumoniae*	CGTCGTATCTCCGCACTCG CGGGTACATCTCGTCTTCG	53	501	[16]
glp	*S. aureus*	CTAGGAACTGCAATCTTAATCC TGGTAAAATCGCATGTCCAATTC	55	500	[17]
ppsA	*P. aeruginosa*	GGTCGCTCGGTCAAGGTAGTGG GGGTTCTCTTCTTCCGGCTCGTAG	55	989	[18]
gyd	*E. faecalis*	CAAACTGCTTAG CTCCAATGGC CATTTCGTTGTCATACCAAGC	52	495	[19]

2.6. Virulence associated gene amplification

The PCR screening of bacterial isolates from each group was a meticulous process. We screened for the presence of various virulence genes, such as *aer* (aerolysin), *alt* (cytotonic heat-labile

enterotoxin), *lip* (lipase), *ast* and *hlyA* (hemolysin A) gene in *A. hydrophila*; *aer* (aerolysin), *alt* (cytotonic heat-labile enterotoxin), *lip* (lipase), *ast* and *hlyA* (hemolysin A) gene in *A. veronii*; *hlyA* (hemolysin A), *ehxA* (enterohemolysin), *stx1*, *stx2* and *eae* gene in *E. coli*; *entB* (enterobactin), *fimH*, *magA*, *rmpA* and *uge* (UDP galacturonate 4-epimerase) gene in *K. pneumoniae*; *hlb* (hemolysin β), *seb*, *sec*, *sed* and *see* gene in *S. aureus*; *exoS*, *lasB*, *toxA*, *oprL* and *phzM* gene for *P. aeruginosa*; *gelE*, *cytA*, *esp*, *hyl* and *efaA* gene for *E. faecalis*. The details of the primers used for the detection of virulence genes are listed in Table 2. The PCR condition was set as mentioned earlier. As discussed in the above section, the amplified products were sequenced with utmost precision in forward and reverse directions. The nucleotide sequences thus obtained were trimmed, refined and submitted to "BankIt-NCBI-NIH" (www.ncbi.nlm.nih.gov/WebSub/).

Table 2. List of primers of virulence gene used in this study.

Target gene	Bacterial isolate	Primer sequences (5′–3′)	Annealing temperature (°C)	Amplicon size (bp)	Ref.
aer	*A. hydrophila*	TGTCGGSGATGACATGGAYGTG CCAGTTCCAGTCCCACCACTTCA	62	720	[20]
alt	*A. hydrophila*	TGACCCAGTCCTGGCACGGC GGTGATCGATCACCACCAGC	64	442	[21]
ast	*A. hydrophila*	TCTCCATGCTTCCCTTCCACT GTGTAGGGATTGAAGAAGCCG	63	331	[22]
lip	*A. hydrophila*	ATCTTCTCCGACTGGTTCGG CCGTGCCAGGACTGGGTCTT	55	382	[23]
hlyA	*A. hydrophila*	GGCCGGTGGCCCGAAGATACGGG GGCGGCGCCGGACGAGACGGG	55	597	[24].
aer	*A. veronii*	CCAGTTCCAGTCCCACCACTTCA CAAGAACAAGTTCAAGTGGCCA	60	403	this study
alt	*A. veronii*	TGACCCAGTCCTGGCACGGC GGTGATCGATCACCACCAGC	64	442	[21]
ast	*A. veronii*	TCTCCATGCTTCCCTTCCACT GTGTAGGGATTGAAGAAGCCG	63	331	[22]
lip	*A. veronii*	ATCTTCTCCGACTGGTTCGG CCGTGCCAGGACTGGGTCTT	55	382	[23]
hlyA	*A. veronii*	GGCCGGTGGCCCGAAGATACGGG GGCGGCGCCGGACGAGACGGG	55	597	[24]
hlyA	*E. coli*	GTCTGCAAAGCAATCCGCTGCAAAT AAA CTGTGTCCACGAGTTGGTTGATTAG	58	561	[25]
ehxA	*E. coli*	GCATCATCAAGCGTACGTTCC AATGAGCCAAGCTGGTTAAGCT	54	534	[26]
stx1	*E. coli*	ATAAATCGCCATTCGTTGACTAC AGAACGCCCACTGAGATCATC	65	180	[27]

Continued on next page

Target gene	Bacterial isolate	Primer sequences (5′–3′)	Annealing temperature (°C)	Amplicon size (bp)	Ref.
stx2	*E. coli*	GGCACTGTCTGAAACTGCTCC TCGCCAGTTATCTGACATTCTG	65	255	[27]
eae	*E. coli*	GACCCGGCACAAGCATAAGC CCACCTGCAGCAACAAGAGG	65	384	[27]
entB	*K. pneumoniae*	GATGAAGACGATACCGTGC ACCGAATCCAGACCGTAGTC	55	391	[28]
fimH	*K. pneumoniae*	TGGTGGTCGACCTCTCCACGCAGAT TTTTTGCC TCAGCTGAACGCCTATCCCCTGCGC CGGCGAGGCGG	62	576	[29]
uge	*K. pneumoniae*	GATCATCCGGTCTCCTGTA TCTTCACGCCTTCCTTCACT	51	534	[30]
magA	*K. pneumoniae*	GGTGCTCTTTACATCATTGC GCAATGGCCATTTGCGTTAG	53	1282	[31]
rmpA	*K. pneumoniae*	ACTGGGCTACCTCTGCTTCA CTTGCATGAGCCATCTTTCA	50	535	[32]
hlb	*S. aureus*	GTGCACTTACTGACAATAGTGC GTTGATGAGTAGCTACCTTCAGT	52	309	[33]
sea	*S. aureus*	GAAAAAAGTCTGAATTGCAGGGAA CA CAAATAAATCGTAATTAACCGAAGG TTC	52	560	[34]
seb	*S. aureus*	GTATGGTGGTGTAACTGAGC CCAAATAGTGACGAGTTAGG	57	164	[35]
sec	*S. aureus*	AGATGAAGTAGTTGATGTGTATGG CACACTTTTAGAATCAACCG	57	451	[35]
see	*S. aureus*	AGGTTTTTTCACAGGTCATCC CTTTTTTTTCTTCGGTCAATC	57	209	[35]
exoS	*P. aeruginosa*	CGTCGTGTTCAAGCAGATGGTGCTG CCGAACCGCTTCACCAGGC	55	444	[36]
lasB	*P. aeruginosa*	GGAATGAACGAAGCGTTCTCCGAC TGGCGTCGACGAACACCTCG	65	284	[36]
oprL	*P. aeruginosa*	ATGGAAATGCTGAAATTCGGC CTTCTTCAGCTCGACGCGACG	55	504	[37]
toxA	*P. aeruginosa*	GACAACGCCCTCAGCATCACC AGC CGCTGGCCCATTCGCTCC AGCGCT	55	396	[38]
phzM	*P. aeruginosa*	ATGGAGAGCGGGATCGACAG ATGCGGGTTTCCATCGGCAG	54	875	[39]
gelE	*E. faecalis*	TATGACAATGCTTTTTGGGAT AGATGCACCCGAAATAATATA	56	213	[40]

Continued on next page

Target gene	Bacterial isolate	Primer sequences (5′–3′)	Annealing temperature (°C)	Amplicon size (bp)	Ref.
cytA	*E. faecalis*	ACTCGGGGATTGATAGGC GCTGCTAAAGCTGCGCTT	56	688	[40]
esp	*E. faecalis*	AGATTTCATCTTTGATTCTTGG AATTGATTCTTTAGCATCTGG	56	510	[40]
hyl	*E. faecalis*	ACAGAAGAGCTGCAGGAAATG GACTGACGTCCAAGTTTCCAA	56	276	[40]
efaA	*E. faecalis*	GCCAATTGGGACAGACCCTC CGCCTTCTGTTCCTTCTTTGGC	56	688	[41]

2.7. Experimental challenge and LD_{50} determination

The LD_{50} (median lethal dose) test estimates the dose of a test substance that produces 50% death in a given species of animals [42]. To determine the LD_{50}, 800 healthy fingerlings (mean ± SD weight = 22.0 ± 4.3 g) of *L. rohita* were acclimatized for 15 days in an FRP tank with proper aeration and fed twice daily with 2% of their body weight. After acclimatization, ten fish were randomly taken and distributed in seven 200 L tanks assigned for the challenged study and one tank for the control group. All experiments are designed in a triplicate manner. Considering their prevalence frequency, virulence potential and statistical significance, *A. hydrophila* and *P. aeruginosa* were explicitly chosen for a challenge study to determine their LD_{50}. The bacterial isolates were sub-cultured in TSB overnight at 37 °C and then centrifuged at 5000 rpm for 5 min. After centrifugation, the supernatant was discarded and the bacterial pellet was washed twice with sterile normal saline water. Then it was resuspended and serially diluted upto 10^{-7} in sterile normal saline water. Afterward, the Colony-Forming Units per milliliter (CFU/mL) of bacterial suspension was determined by the spread-plate method. 200 µL inoculum of *A. hydrophila* were prepared for intraperitoneal injection with different concentrations of 2.0×10^8, 2.0×10^7, 2.0×10^6, 2.0×10^5, 2.0×10^4, 2.0×10^3 and 2.0×10^2 CFU/mL in respective group. Similarly, 200 µL bacterial suspension of *P. aeruginosa* was intraperitoneally injected with different concentrations of 2.5×10^8, 2.5×10^7, 2.5×10^6, 2.5×10^5, 2.5×10^4, 2.5×10^3 and 2.5×10^2 CFU/mL in the respective group. The control group was intraperitoneally injected with 200 µL of normal saline. Afterwards, fish mortality was recorded for 168 h to calculate the percentage of cumulative mortality and the LD_{50} value was determined by log-probit analysis. The injected bacterial isolates become reisolated and identified from the liver, kidney and blood of moribund fish to confirm Koch's postulates. All the statistical analyses were done using SPSS version 16.0 (SPSS Inc. Released 2007, Chicago, SPSS Inc).

2.8. Histopathology study

The tissue samples, including gill, intestine, kidney and liver from infected fish were preserved in 10% NBF, cut into small blocks, and dehydrated through a series of 30%, 50%, 70%, and 90% absolute alcohol concentrations. Then, all the samples were cleared in xylene and embedded into paraffin following the infiltration process (Leica EG1140H, Germany). The tissue blocks were trimmed and sectioned at 5 µm thickness using a rotary microtome (Leica RM2025, Germany) followed by a double staining technique with hematoxylin and eosin [43]. Stained sections were

mounted with DPX, and microphotographs were taken after a thorough observation under the light microscope.

2.9. Statistical analysis

The Kruskal-Wallis H test is used to test the null hypothesis that all k-independent samples (i.e., seven bacterial groups) came from populations having equal mean values of virulence. The alternative hypothesis is that at least one population varies in terms of mean values of virulence. The Kruskal-Wallis H test is a robust rank-based test for the k-sample problem as an alternative to the parametric approaches. This is an extremely useful test when the assumptions of normality do not hold or the population variances are not equal. The significant Kruskal-Wallis (H-value) result was further analyzed with Dunn's test (post-hoc) to find out the exact difference in virulence mean rank value between bacterial groups [44]. All the statistical analyses were done using SPSS version 16.0 (SPSS Inc. Released 2007, Chicago, SPSS Inc).

3. Results

3.1. Frequency of bacterial isolates

A total of 171 diseased fish were collected for detection of pathogenic bacteria. Initially, the bacterial isolates were screened using differences in colony morphology. These isolates were transferred into different selective media and further checked through solid-phase hemolysis. This comprehensive approach identified 36 pathogenic pure bacterial isolates, a diverse range of 7 different genera, obtained from the diseased fish. The species of these 36 isolates were confirmed by biochemical characterization, 16S rRNA amplification, sequencing, BLAST-N search (http://www.ncbi.nlm.nih.gov/BLAST) and phylogenetic analysis. The species of the isolates were further reconfirmed utilizing housekeeping genes. The bacterial isolates from the diseased fish consisted of *A. hydrophila* (25%), *K. pneumoniae* (6%), *E. coli* (5%), *S. aureus* (6%), *A. veronii* (22%), *P. aeruginosa* (22%), and *E. faecalis* (14%). The representatives of prevalent bacterial isolates were organized in ascending order: *A. hydrophila*, *A. veronii*, *P. aeruginosa*, *E. faecalis*, *S. aureus* and *E. coli*. This study's most predominant genus was *Aeromonas spp*., accounting for 47% of the isolates.

3.2. Biochemical characterization

Gram staining results showed that *E. faecalis* and *S. aureus* tested for Gram-positive while *A. hydrophila, A. veronii, K. pneumoniae, P. aeruginosa*, and *E. coli* isolates were Gram-negative. Major biochemical tests like the ONPG (β-galactosidase) test, Lysine utilization test, Urease test, H_2S production and Voges-Proskauer's test of studied isolates were consistent with previous findings of the particular species. Overall, the biochemical tests for selected isolates were counter-foiled with reference strains, as illustrated in Figure 2. In solid-phase hemolysis assay, 28 isolates demonstrated α-hemolysis while 8 isolates exhibited β-hemolysis.

3.3. DNA sequencing and phylogenetic analysis

The 16S rRNA sequences and housekeeping genes of bacterial isolates were submitted to "NCBI-BankIt", and a GeneBank accession number was generated (Table 3). Bacterial species such as *A. hydrophila*, *A. veronii*, *P. aeruginosa*, *K. pneumoniae*, *E. faecalis*, *S. aureus* and *E. coli* were confirmed through BLAST-N search using the 16S rRNA sequences and housekeeping gene sequences. Based on 16S rRNA sequences, NJ tree (Figure 3) was constructed, revealing that the isolated strains RI13, RI5, RI8, A4, C12SH, C10SH, C18SH, C15SH, and C17SH form a cluster with *A. hydrophila* (cluster-1) with a high bootstrap value. Two isolated strains, CIFRIA5 and A5 form a cluster with *S. aureus* (cluster-2); however, they form two different clades. Similarly, strains NONS3, PS3, PS4, PS5, and PS7 cluster with *E. faecalis* (cluster-3). Though they cluster together, they form different clades. Further, eight isolated strains, P2HA, JayT1, JayT3, JayT7, JayT9, JayT12, JayT13, and SM673 cluster with *P. aeruginosa* (cluster-4) with a high bootstrap value. Two isolates, A2KP and A2, form a cluster with *K. pneumoniae* with a bootstrap value of 99 (cluster-5), and two isolates A3 and K5, cluster with *E. coli* (cluster-6). Both clusters emerged from a single node, which strongly indicates that *K. pneumoniae* and *E. coli* are closely related evolutionarily, as they both belong to the Enterobacteriaceae family.

Figure 2. Heat map for a comparative biochemical test result of isolated bacteria. Comparative biochemical test of seven groups of bacterial isolates from diseased *L. rohita* against published biochemical data of respective isolate; green color represents positive test; red color represents negative test and black color denotes for not determined (ND).

Table 3. NCBI accession numbers and Gene profile of bacterial isolates from diseased *L. rohita.*

Bacterial isolates	Strain	Gene Accession number of 16S rRNA gene	Amplified housekeeping gene	Gene Accession number of housekeeping gene	Virulence genes Screened for	Virulence genes Amplified for	Gene Accession number of Virulence genes
A. hydrophila	RI13, RI5, RI8, A4, C12SH, C10SH, C18SH, C15SH, C17SH	ON075438, ON075436, ON075437, MZ378784, OM010339, OL739291, OK284540, OL719292, OL719071	*gyrB*	ON209428	*aer*, *alt*, *lip*, *hlyA*, *ast*	*aer*, *alt*, *lip*, *hlyA*	OL763272 ON209426 ON209427 OM045779
A. veronii	P42KHN, C16SH, P21KHN, GO2, GO1, CCECW6, GO4, GO3	OK284516, OK284460, OK284515, ON025208, ON025205, ON025207, ON141893, ON141892	*rpoB*	ON921218	*aer*, *alt*, *lip*, *hlyA*, *ast*	*aer*	OP035837
E. faecalis	P36KHN, PS3, PS4, PS5, PS7	OK284461, ON092681, ON092630, ON092640, ON092685	*gyd*	OP080332	*gelE*, *cytA*, *esp*, *hyl*, *efaA*	*gelE*	ON993363
E. coli	A3, K5	MZ377305, ON679519	*recA*	ON959265	*hlyA*, *ehxA*, *stx1*, *stx2*, *eae*	*hlyA*	OK318733
K. pneumoniae	deswaKP, deswaA2	MZ377304, ON197774	*rpoB*	ON959266	*fimhA*, *uge*, *entB*, *magA*, *rmpA*	*fimhA*, *uge*, *entB*	ON974991 OP009353 OP009352
P. aeruginosa	JayT1, JayT3, JayT7, JayT9, JayT12, JayT13, SM673, P2HA	OL409183, ON024353, ON024358, ON024342, ON024351, ON024356, ON024375, ON024754	*ppsA*	ON983971	*exoS*, *lasB*, *toxA*, *oprL*, *phzM*	*exoS*, *lasB*, *toxA*, *oprL*, *phzM*	ON983972 ON209429 ON983973 ON993362 ON993361
S. aureus	CIFRIA-5, A5	OL989453, OM021851	*glp*	ON974990	*hlb*, *seb*, *sec*, *sea*, *see*	*hlb*	OP009354

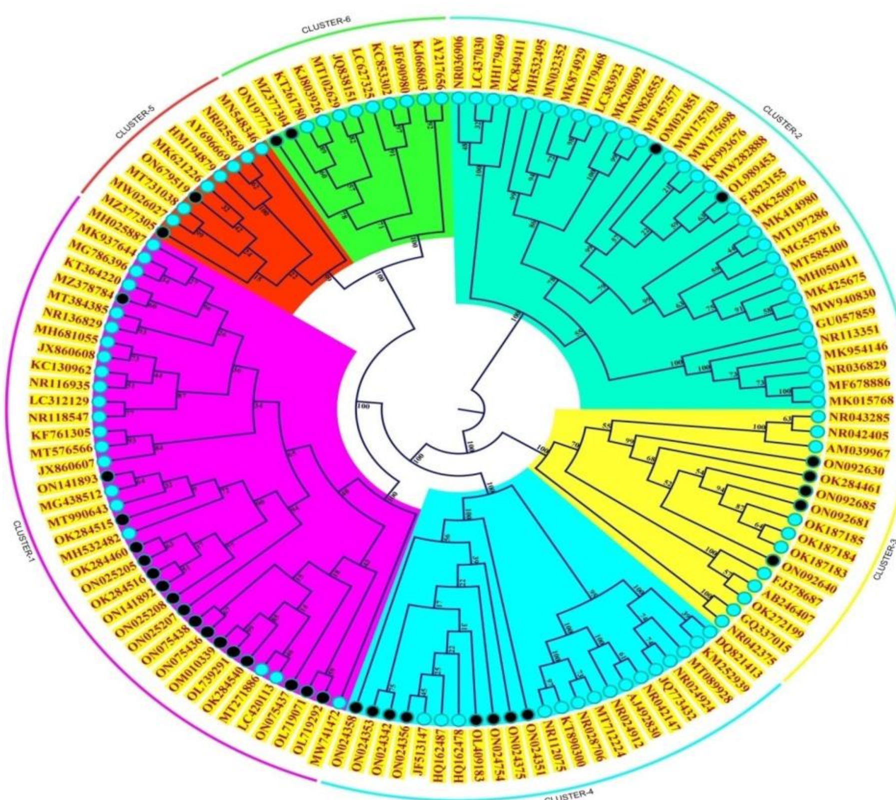

Figure 3. Phylogenetic analysis of isolated bacteria based on 16S rRNA nucleotide sequences. Phylogenetic analysis was inferred following the Neighbor-Joining (NJ) method by CLC Genomics Workbench 21.0.5 software. The numbers next to the branches indicate percentage values for 1000 bootstrap replicates. Black circles represent our studied bacterial isolates obtained from diseased *L. rohita*; cluster-1, cluster-2, cluster-3, cluster-4, cluster-5, and cluster-6 correspond to *Aeromonas sp.*, *Staphylococcus sp.*, *Enterococcus sp.*, *Pseudomonas sp.*, *Escherichia sp.*, and *Klebsiella sp.*, respectively.

Additional BLAST analysis was conducted using selective housekeeping genes from various species, revealing a complete match with strains of *A. veronii, P. aeruginosa, S. aureus, E. faecalis, K. pneumoniae, A. hydrophila* and *E. coli* with identities ranging from 99% to 100%. These genes are represented in the agarose gel images shown in Figure 4. This comprehensive analysis strongly correlates with the findings derived from the analysis of the 16S rRNA sequences, reinforcing the validity of the results. Furthermore, phylogenetic trees were constructed utilizing different housekeeping genes (rpoB: ON921218; ppsA: ON983971; glpF: ON974990; gyd: OP080332; rpoB: ON959266; gyrB: ON209428), forming clusters consistent with their respective species (Figure S5).

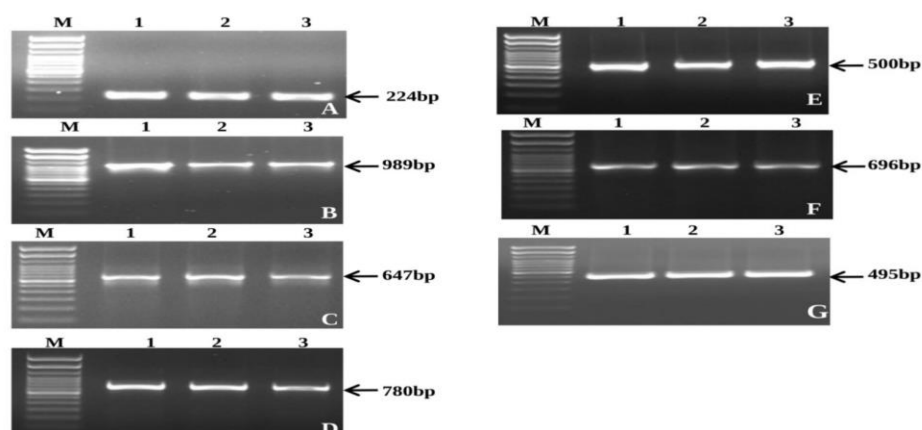

Figure 4. Gel image of PCR amplified products of housekeeping genes. (A) *rpoB* gene of *A. veronii*, Lane 1–3 indicates amplicon of *rpoB* gene with product size ~224 bp (B) *ppsA* gene of *P. aeruginosa,* Lane 1–3 indicates amplicon of *ppsA* gene with product size ~989 bp (C) *rpoB* gene of *K. pneumoniae,* Lane 1–3 indicates amplicon of *rpoB* gene with product size ~647 bp (D) *recA* gene of *E. coli,* Lane 1–3 indicates amplicon of *recA* gene with product size ~780 bp (E) *glp* gene *of S. aureus* , Lane 1–3 indicates amplicon of *glp* gene with product size ~500 bp (F) *gyrB* gene *of A. hydrophila*, and Lane 1–3 indicates amplicon of *gyrB* gene with product size ~696 bp (G) *gyd* gene *of E. faecalis.* Lane 1–3 indicates amplicon of *gyd* gene with product size ~495 bp M: Molecular weight marker (100 bp).

3.4. Virulence gene amplification

According to Snieszko (1972) [45], to develop a disease, the host's physiological status and virulence potentiality of a pathogen are invariably interrelated. In this investigation, seven groups of bacterial isolates (*A. hydrophila*, *A. veronii*, *P. aeruginosa*, *K. pneumoniae*, *E. faecalis*, *S. aureus*, and *E. coli*) were examined for the presence of five virulence genes in each, resulting in the screening of a total of 35 virulence genes among all 36 bacterial isolates. The profiles of amplified PCR products for virulence genes are depicted in Figure 5. Among nine strains of *A. hydrophila*, virulence gene *alt* was detected in 7 strains, *lip* in 8 strains, *hlyA* in 6 strains, *aer* in 4 strains, while the *ast* gene was positive in none of the isolates. Similarly, only 3 strains of *A. veronii* were detected positive for only one gene, *aer*, with product size 403 bp. Among eight strains of *P. aeruginosa*, only 3 strains JayT1, JayT3, and JayT13 were amplified positive for all five virulent genes like *lasB*, *exoS*, *toxA*, *oprL* and *phzM*. Both strains A2 and A2KP of *K. pneumoniae* were amplified for *entB*, *fimH* and *uge* at their specific product size 391 bp, 576 bp and 538 bp respectively. Both *S. aureus* and *E. coli* strains were detected positive for one virulent gene *hlb* and *hlyA* respectively. Five strains of *E. faecalis* were screened positive for only one virulence factor, *gelE*, with a product size of 213 bp. The BLAST-N search validated the presence of virulence genes under examination, which were submitted to NCBI. The corresponding gene bank accession numbers are listed in Table 3.

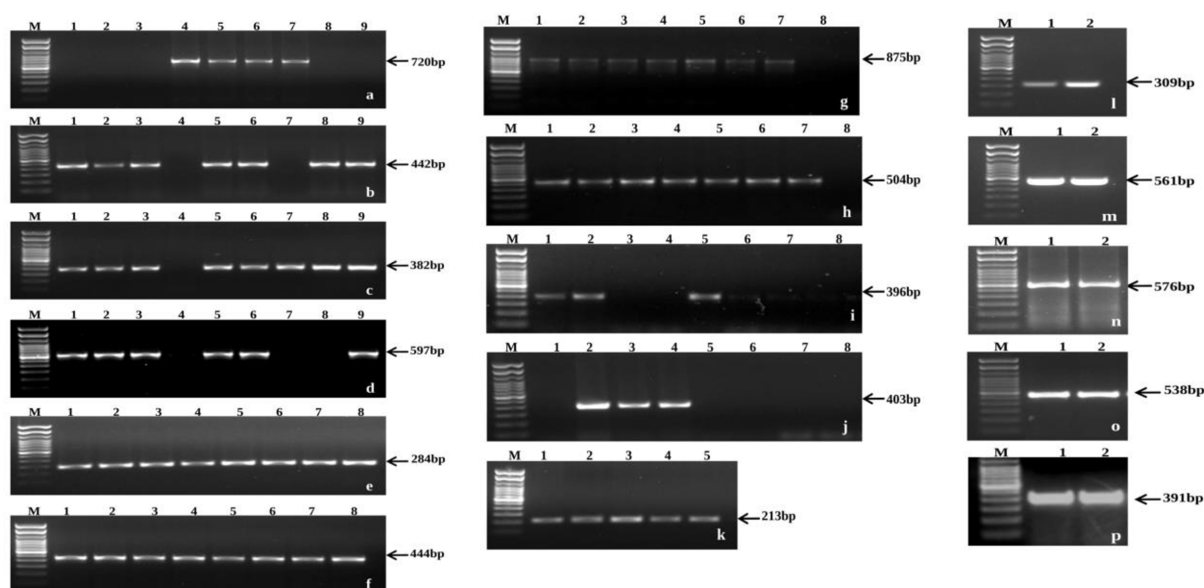

Figure 5. Gel image of PCR amplified products of virulence genes. (a) *aer* gene of *A. hydrophila*, Lane 1–9 indicates amplicon of *aer* gene with product size ~720bp (b) *alt* gene of *A. hydrophila*, Lane 1–9 indicates amplicon of *alt* gene with product size ~442 bp (c) *lip* gene of *A. hydrophila*, Lane 1–9 indicates amplicon of *lip* gene with product size ~382 bp (d) *hlyA* gene of *A. hydrophila*, Lane 1–9 indicates amplicon of *hlyA* gene with product size ~597bp (e) *lasB* gene of *P. aeruginosa*, Lane 1–8 indicates amplicon of *lasB* gene with product size ~284 bp (f) *exoS* gene of *P. aeruginosa*, Lane 1–8 indicates amplicon of *exoS* gene with product size ~444 bp (g) *phzM* gene of *P. aeruginosa*, Lane 1–8 indicates amplicon of *phzM* gene with product size ~875 bp (h) *oprL* gene of *P. aeruginosa*, Lane 1–8 indicates amplicon of *oprL* gene with product size ~504 bp (i) *toxA* gene of *P. aeruginosa*, Lane 1–8 indicates amplicon of *toxA* gene with product size ~396 bp (j) *aer* gene of *A.veronii*, Lane 1–8 indicates amplicon of *aer* gene with product size ~403 bp (k) *gelE* gene of *E. faecalis*, Lane 1–8 indicates amplicon of *gelE* gene with product size ~213 bp. (l) *hlb* gene of *S. aureus*, Lane 1–2 indicates amplicon of *hlb* gene with product size ~309 bp (m) *hlyA* gene of *E.coli*, Lane 1–2 indicates amplicon of *hlyA* gene with product size ~875 bp (n) *fimH* gene of *K. pneumoniae*, Lane 1–2 indicates amplicon of *fimH* gene with product size ~576 bp (o) *uge* gene of *K. pneumoniae*, Lane 1–2 indicates amplicon of *uge* gene with product size ~538 bp (p) *entB* gene of *K. pneumoniae*, and Lane 1–2 indicates amplicon of *entB* gene with product size ~391 bp M: Molecular weight marker (100 bp).

3.5. Statistical analysis

The mean virulence rank value (Table 4) is highest in *P. aeruginosa* followed by *K. pneumoniae*, *A. hydrophila*, *S. aureus*, *E. faecalis*, *E. coli* and *A. veronii* isolates. The result of the Kruskal-Wallis test (H-value) shows a statistically significant difference ($p < 0.001$). The result of Dunn's test (post hoc) between or among bacterial groups shows a statistically significant difference in mean rank virulence value exists between *A. hydrophila* vs. *A. veronii* ($p < 0.001$) and *A. veronii* vs. *P. aeruginosa* ($p < 0.001$).

Table 4. Result of Mean Rank value and Kruskal-Wallis test (H-value) across Bacterial groups.

Isolates	n	Mean Rank	Kruskal-Wallis H test	*p*	df
A. hydrophila	9	23.56	29.153	<0.001	6
A. veronii	8	7.00			
P. aeruginosa	8	30.81			
S. aureus	2	11.50			
E. faecalis	5	11.50			
E. coli	2	11.50			
K. pneumoniae	2	24.00			
Post-Hoc (Dunn's) test					
Comparison between group			*p*		
A. hydrophila vs. *A. veronii*			<0.001		
A. veronii vs. *P. aeruginosa*			<0.001		

3.6. Experimental challenge and LD50 determination

During the challenged study 100% fish mortality was observed within 72 h in two groups of *L. rohita* challenged by *A. hydrophila* (C18SH) and *P. aeruginosa* (JayT3) with final concentrations of 2.0×10^8 and 2.5×10^8 CFU/mL respectively. No mortality was found in the control group after seven days of post-infection. The challenged fish exhibited similar signs and symptoms such as hemorrhaging spots and reddening around the injection site and over the belly surface, intestinal fluid accumulation and extended belly portion. The log-probit analysis of *L. rohita* is presented in Figure 6. The calculated LD_{50} value with 95% C.I. of three bacterial isolates has resulted as follows: 1.5×10^4 for *A. hydrophila* (C18SH) and 6.1×10^4 for *P. aeruginosa* (JayT3). The detailed log-probit regression equation was tabulated in Table S2.

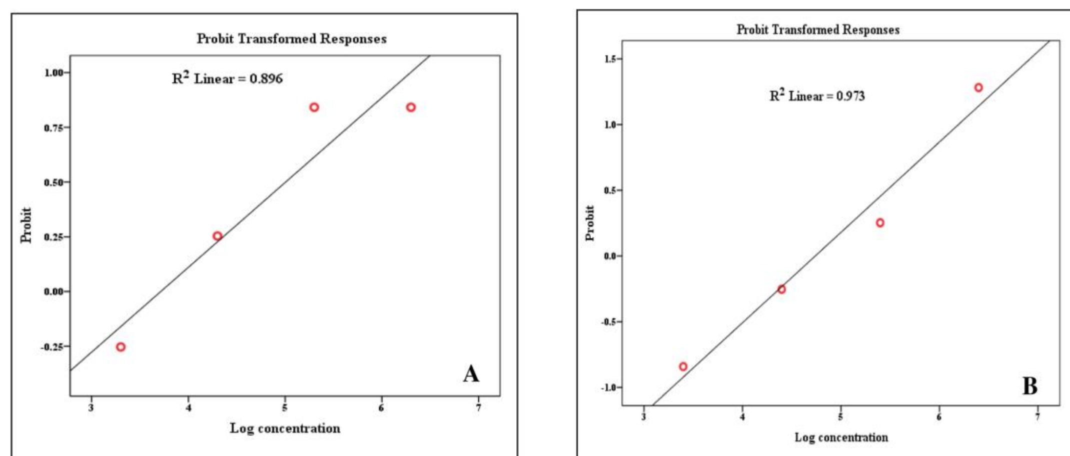

Figure 6. Determination of LD_{50} value by log-probit analysis. (A) Log-probit model regression lines for log concentration versus probit mortality for *L. rohita* following 168 h exposure by *A. hydrophila* (C18SH) intraperitoneally (B) Log-probit model regression lines for log concentration versus probit mortality for *L. rohita* following 168 h exposure by *P. aeruginosa* (JayT3) intraperitoneally.

3.7. Histopathology study

Histopathological observation shows that organs and tissues exhibited extensive hemorrhagic spots, multifocal lesions and infiltration of inflammatory cells. In the intestinal villi, there was loose, broken and intensive degeneration of epithelial submucosa. Thick and clubbed primary and secondary gill lamellae with swollen distal tips were observed in the gill section. A transverse section (TS) of the liver tissue exhibited degenerative changes, hepatocyte hypertrophy, blood congestion and pathologic lesions. Moreover, the histological section of the kidney manifested mainly vacuolation of tubular epithelial cells, disorganization of renal tubules and glomerular atrophy (Figure 7).

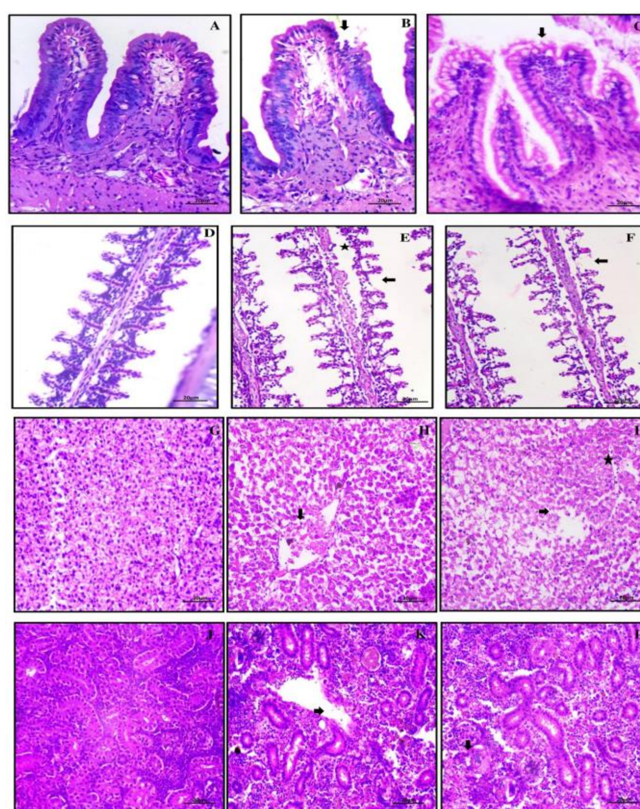

Figure 7. Photomicrograph of histological features of intestinal tissue (A–C), gill (D–F), liver tissue (G–I) and renal tissue (J-L) of *L. rohita* (10 × 40X). (A) Intestine of controlled fish. (B) Intestine of challenged fish (*A. hydrophila*), degeneration of epithelial submucosa [arrow]. (C) Intestine of challenged fish (*P. aeruginosa*), architectural and structural alterations [arrow], degeneration of epithelial submucosa [arrow] and broken tips of villi [star]. (D) Gill of controlled fish. (E) Gill of challenged fish (*A. hydrophila*), degeneration and disorientation of secondary gill lamellae [arrow]. (F) Gill of challenged fish (*P. aeruginosa*), and lost or sunken of secondary gill lamellae [arrow]. (G) Liver of controlled fish. (H) Liver of challenged fish (*A. hydrophila*), and degenerative changes [arrow]. (I) Liver of challenged fish (*P. aeruginosa*), hepatocyte hypertrophy (arrow) and blood congestion [star]. (J) Kidney of controlled fish. (K) Kidney of challenged fish (*A. hydrophila*), and vacuolation formation [arrow]. (L) Kidney of challenged fish (*P. aeruginosa*), and glomerular atrophy [arrow].

4. Discussion

Diseases are the major challenge in the sustainable development of aquaculture, which strives to meet the global demand for nutritional security and provide livelihoods to millions of people. To address this, it is crucial to study the prevalence of infections in fish to better understand the epidemiology of emergent pathogens. The present study was designed to comprehensively investigate the fish bacterial pathogens associated with infection and mortality in the Hooghly district of West Bengal, India. The affected fish were initially examined for clinical signs, followed by identifying bacterial pathogens through biochemical tests, 16S rRNA sequencing and species-specific gene amplification. Most of the identified fish pathogens were Gram-negative, belonging to the families Enterobacteriaceae, Aeromonadaceae, and Pseudomonadaceae, and Gram-positive bacteria from Staphylococcaceae and Enterococcaceae. The ponds, lakes and natural water bodies in the Hooghly district of West Bengal are at risk of direct or indirect contamination from various sources, including domestic sewage, agricultural runoff, drainage effluents and fecal pollution. These contaminants can potentially foster and elevate the load of pathogenic bacteria within these reservoirs.

In this study, we isolated and characterized bacterial species, *viz*., *A. hydrophila, A. veronii, P. aeruginosa, K. pneumoniae, E. faecalis, S. aureus* and *E. coli* from diseased fishes from different areas of Hooghly, West Bengal. *Aeromonas spp.* is a Gram-negative, opportunistic zoonotic pathogen and is recognizable for septicemia, ulcerative and hemorrhagic diseases in freshwater as well as marine fish. It belongs to the genus *Aeromonas*, class Gammaproteobacteria, order Aeromonadales and family Aeromonadaceae [46]. Several research findings suggested that *Aeromonas spp*. is the significant causative agent for mass fish mortality posing a concerning threat to the aquaculture industry [47,48]. Moreover, similar studies observed that *K. pneumoniae* isolated from diseased Indian Major Carps in West Bengal imposed a significant constraint on aquaculture systems [49]. *S. aureus* is another potential superbug of the fishery industry and has been documented in several countries like Japan, India, China, Brazil, Iran etc. [50,51]. Further, pseudomonads are the most challenging fish pathogen caused by ulcerative syndrome and hemorrhagic septicemia [52].

The bacterial isolates were screened using different selective media and were characterized biochemically. Slight biochemical variations were observed in our studied bacterial strains compared to their reference strains, possibly due to different geographical locations of sample collection sites [53]. Further, molecular identification of the strains through 16S rRNA sequencing was conducted, as it is difficult to confirm the bacterial species based on classical phenotypic features [54]. 16S rRNA PCR based sequencing is a rapid, reliable and useful tool for identifying microbes. Further, the phylogenetic cladogram of 16S rRNA sequencing revealed that each bacterial isolate constructs its own clade and its high bootstrap value reflected that they are closely associated with their sister group and represent a common ancestor. Thus, phylogenetic relatedness could be used as a molecular chronometer to establish an evolutionary relationship at the genus level and sometimes at the species level [55]. Furthermore, several housekeeping genes were screened for species-level identification of bacterial isolates. Bacterial hemolytic activity is considered one of the markers of pathogenic bacteria and for the discrimination of virulent strains from avirulent ones [48,56]. The isolates were examined for hemolysis assays to confirm the pathogenicity of the strains in the present study. All the identified strains showed either a typical characteristic, i.e., α or β-hemolytic activity on a blood agar plate which was further supported by the detection of several hemolysin genes. Earlier studies also confirmed the presence of the hemolysin gene in several pathogenic bacteria *viz*., *Vibrio cholerae* [57], *Edwardsiella tarda* [58,59], *Streptococcus iniae* [60], and *V. parahaemolyticus* [61] which

contributes to the pathogenicity of the bacterial strain.

Sixteen virulence genes detected in this study among all bacterial isolates, were responsible for the expression of bacterial pathogenicity. A bacterium has innumerable array of invading, masking and escaping mechanisms such as the presence of capsule, flagellum, adhesion protein, exotoxin, enterotoxin, iron acquisition mechanisms, quorum sensing activity, and secretion systems (Type-II, III, IV, VI) against the host defense mechanism [47,61]. In our study, *entB*, *fimH* and *uge* genes were detected in strains A2KP and A2 (*K. pneumoniae*), which supported the previous findings by Remya et al., and Sivaraman et al. [62,63]. *K. pneumoniae* expressed one to several siderophores for scavenging Fe^{3+} from host proteins, whereas *entB* encodes for Enterobactin (core siderophore), *fimH* expressed as adhesion type 1 fimbriae. Regué et al. mentioned that those strains of *K. pneumoniae* harboring the *uge* gene were more virulent than the isolates without the *uge* gene [64]. The four isolates (*A. hydrophila*) in our study harbor the *aer* gene that codes for aerolysin and is a critical pore-forming enterotoxin that plays a vital role in the pathogenesis of Aeromonad's infection [65]. We observed that none of the *A. hydrophila* isolates were positive for cytotonic heat-stable enterotoxin (*ast)* [66,67]. On the contrary, cytotonic heat-labile enterotoxin (*alt*) was present in 78% of *A. hydrophila* isolates which is higher than the findings of Nhinh et al. [66]. It was pointed out that tissue damage and fluid accumulation in the intestine are accountable to cytotonic heat-labile enterotoxin (*alt*) in *A. hydrophila* [68,69]. In this study, 67% of the incidence was positive for the *hlyA* gene in *A. hydrophila*, which is lower than frequency 95% reported by Hayati et al. [70]. The *hlyA* gene encodes for hemolysin that influences red blood cell (RBC) lysis in various host species [71,72]. Another essential virulence factor of *A. hydrophila* is the *lip* gene encoding for extracellular lipase that actively alters the host's cell plasma membrane and aggravates the degree of infection [73–75]. In this work, 89% of *A. hydrophila* isolates were positive for the *lip* gene, which is supported by previous studies [48]. Similar to *A. hydrophila*, the *aer* gene also amplified 38% of the strain of *A. veronii* and is in accord with former research studies in cultured catfish and crucian carp [76,77]. A5 and CIFRIA5 (*S. aureus*) isolates from this study harbor the *hlb* gene that produces sphingomyelinase and destabilizes the host's cell plasma membrane integrity [78]. Virulence gene *hlyA* was present in strains A3 and K5 (*E. coli*). The presence of the *hlyA* gene in *E. coli* was also mentioned in the literature [79,80] and is secreted via a type-I secretory system [81]. The *E. coli* α-hemolysin is caused by urinary tract infection and has a wide range of cytolytic and cytotoxic effects on mammalian cell types [81–83]. In this study, all strains of *P. aeruginosa* were positive for *lasB* and *exoS*, which agrees with previous studies by Alonso et al. and Algammal et al. [84,85]. ExoS is a Type III Secretion System (T3SS) effector protein that induces apoptosis of host cells and inhibits reactive oxygen species (ROS) production, thereby neutralizing the host's first-line of defense against microbial infections [86,87]. Another important virulence factor is *lasB* encoding elastase, which is involved in the degradation of host proteases and inactivation of host immune system components to establish a clinical infection [88]. On the contrary, 88% of *P. aeruginosa* strains were examined positive for *oprL* and *phzM* which agreed with a report of Kenneth and Nowroozi et al. [89,90]. Outer membrane peptidoglycan-associated L- Lipoproteins (PAL) encoded by *oprL* protects the microorganisms against antiseptics and several antimicrobial agents [85,91]. On the other hand, it could be used as a molecular marker for identifying *P. aeruginosa* [92]. *phzM* codes for a phenazine toxin that promotes the survival and colonization of bacteria in adverse conditions where other microorganisms may struggle to survive [93,94]. A lower incidence (37%) was found in the positivity rate of *toxA* gene of *P. aeruginosa* strain which was also observed in Kenneth's study [89]. Previous findings showed that *toxA* encodes for an extracellular product exotoxin A, which is associated with inhibiting host protein biosynthesis [95]. In this study, all strains of *E. faecalis* were

positive for *gelE*, which was supported by previous studies [96,97]. The *gelE*-encoded protein has a positive correlation with biofilm formation in *E. faecalis* [98–100]; in contrast, several research findings unfold that there is no correlation between biofilm formation and the *gelE*-encoded protein [97,101]. From this virulence study, it is understood that for the development of pathogenicity, the bacteria need to adhere, invade, lyse, escape from the host immune response and coordinate the expression of several virulence genes. According to the log-probit analysis, our isolated strain *A. hydrophila* exhibited an LD_{50} dose of 1.5×10^4, indicating a higher pathogenicity level than *P. aeruginosa*. These results are quite significant and more promising than the previous findings by Samayanpaulraj et al. [48].

In the histopathological study, the gill, kidney, liver and intestine exhibited varying degrees of multifocal lesion, hemorrhagic spot and tissue degeneration. Kidney and liver tissues showed similar alterations after the post-challenged study of *L. rohita* with *Acinetobacter baumannii* [102]. Das et al. [49] pointed out the formation of melanomacrophages center in the liver tissue after infection of *L. rohita* by *K. pneumoniae*. Intestinal epithelium tissue disintegrated and loose and necrotic debris was observed within the lumen, which co-relates with the result of Abdelhamed et al. [103] in channel catfish infected with *A. hydrophila*. We noted intestinal fluid accumulation in *L. rohita* infected by *A. hydrophila*, *P. aeruginosa* and *E. coli.* Overall, we affirmed that isolated bacterial strains possess an array of virulence factors that might be responsible for significant histopathological changes and progressive development of multivarious levels of infection.

5. Conclusions

This study represents comprehensive data on identifying and characterizing of pathogenic bacteria virulence profiles obtained from diseased *L. rohita*. Our results reveal that the pseudomonad and aeromonad groups are most potent in terms of their virulence and pathogenicity among 36 bacterial isolates. Importantly, fish health management is a shared responsibility and each stakeholder may have a catalytic role in controlling fish diseases. These data may help the authorities formulate potential prophylactic measures against the dreadful pathogens of the economically important fish in India.

Use of AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

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Conflict of interest

The authors have declared no conflict of interest.

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