



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*; *hlyB* 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

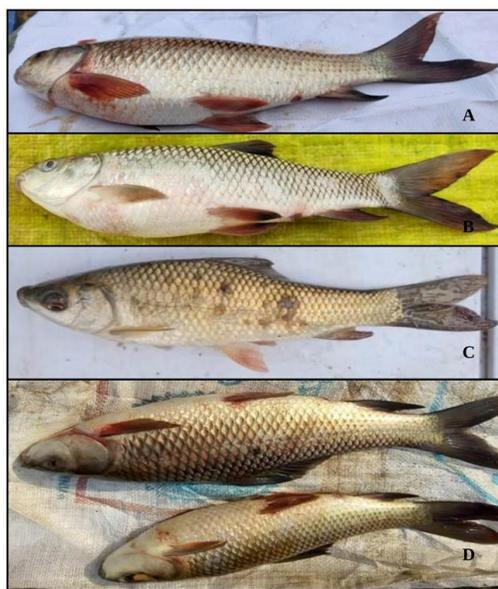
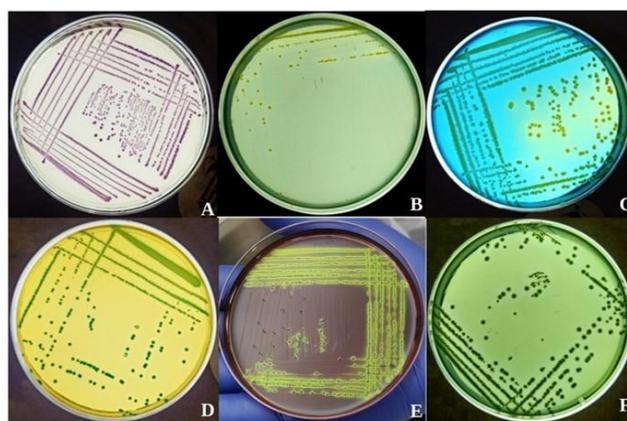
Supplementary files

Table S1. Co-ordinates of sample collection site across District Hooghly, West Bengal, India.

Sample collection Site	Longitude	Latitude	Sample collection Site	Longitude	Latitude
S1	88.092467°E	22.842292°N	S23	88.117475°E	22.829731°N
S2	88.088192°E	22.847025°N	S24	88.086258°E	22.842761°N
S3	88.087022°E	22.846267°N	S25	88.062986°E	22.800481°N
S4	88.240533°E	22.809675°N	S26	88.298689°E	22.788836°N
S5	88.091681°E	22.874303°N	S27	88.300919°E	22.787875°N
S6	88.0916°E	22.874942°N	S28	88.304056°E	22.786381°N
S7	88.091578°E	22.873486°N	S29	88.295072°E	22.787517°N
S8	88.092353°E	22.874275°N	S30	88.295875°E	22.789808°N
S9	88.090978°E	22.873247°N	S31	88.296708°E	22.787622°N
S10	88.275953°E	22.811458°N	S32	87.9914°E	22.839811°N
S11	88.276078°E	22.814161°N	S33	88.088217°E	22.871039°N
S12	88.236953°E	22.81035°N	S34	88.058808°E	22.776358°N
S13	88.2205°E	22.8264°N	S35	88.056997°E	22.777967°N
S14	88.218731°E	22.827919°N	S36	88.058161°E	22.779022°N
S15	88.087758°E	22.871783°N	S37	88.061183°E	22.781192°N
S16	88.093658°E	22.873133°N	S38	88.23121944°E	23.09469167°N
S17	88.086789°E	22.872206°N	S39	88.26171667°E	23.01170556°N
S18	88.111336°E	22.861142°N	S40	87.68576389°E	22.93343056°N
S19	88.110314°E	22.866167°N	S41	87.43709167°E	23.04764444°N
S20	88.114842°E	22.831942°N	S42	87.68486389°E	22.90178333°N
S21	88.109083°E	22.866197°N	S43	87.70510833°E	22.97798611°N
S22	88.107025°E	22.863558°N			

Table S2. Probit analysis result of two selected bacterial isolates.

Bacterial Isolates	LC ₅₀	95% C.I.	Probit regression equation				<i>p</i>
			Intercept	s.e.	β	s.e.	
C18	1.5707×10^4	2.8975×10^4 – 6.3252×10^4	–2.751	0.659	0.656	0.140	0.519
JayT3	6.1300×10^4	1.6485×10^4 – 2.2090×10^5	–3.858	0.828	0.806	0.166	0.937

**Figure S1.** Diseased *Labeo rohita* (A–D) obtained across the District Hooghly, West Bengal, India.**Figure S2.** Photograph of streaked bacterial culture plate of different selective media. (A) *Klebsiella pneumoniae* streaked onto Klebsiella Selective Agar Base culture plate; (B) *Aeromonas veronii* streaked Rimler-Shotts Medium Base culture plate; (C) *Aeromonas hydrophila* streaked onto Aeromonas Selective Agar (BSIBG) culture plate; (D) *Staphylococcus aureus* streaked onto HiCrome Staph Selective Agar culture plate; (E) *Escherichia coli* streaked onto Eosin methylene blue agar (EMB) culture plate; (F) *Aeromonas hydrophila* streaked onto Aeromonas Isolation HiVeg Medium Base culture plate.

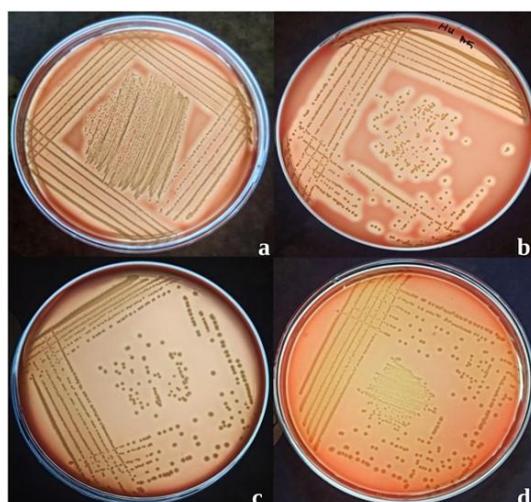


Figure S3. Photograph of streaked blood agar plate supplemented with 2% human RBC. (a and b) Observation of beta hemolysis onto blood agar plate; (a) growth of *A. hydrophila* on blood agar plate, (b) growth of *S. aureus* on blood agar plate. (c and d) Observation of alpha hemolysis onto blood agar plate; (c) growth of *E. coli* on blood agar plate, (d) growth of *K. pneumoniae* on blood agar plate.

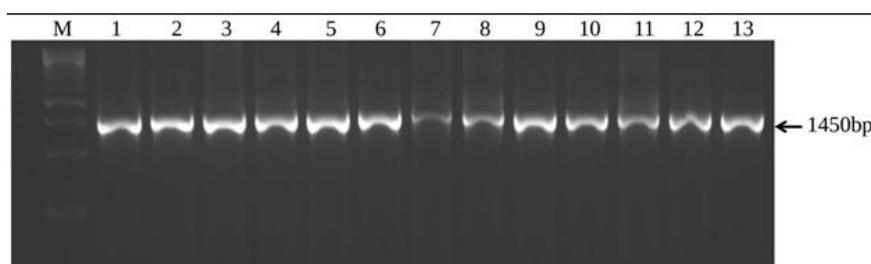
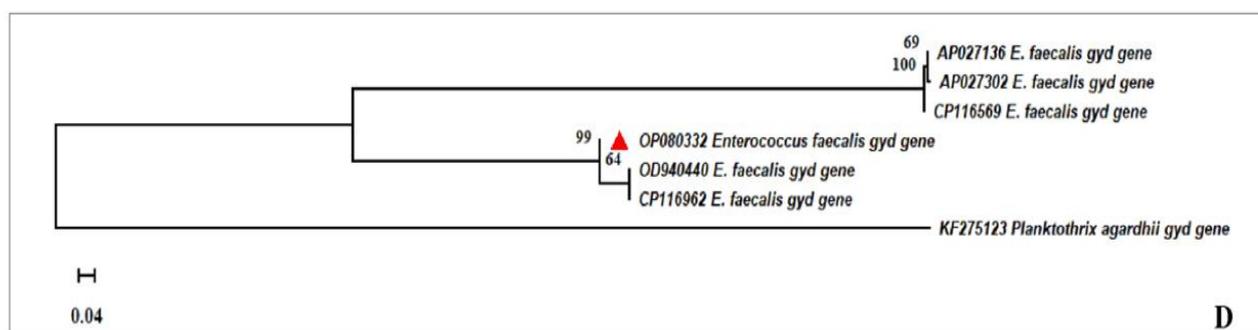
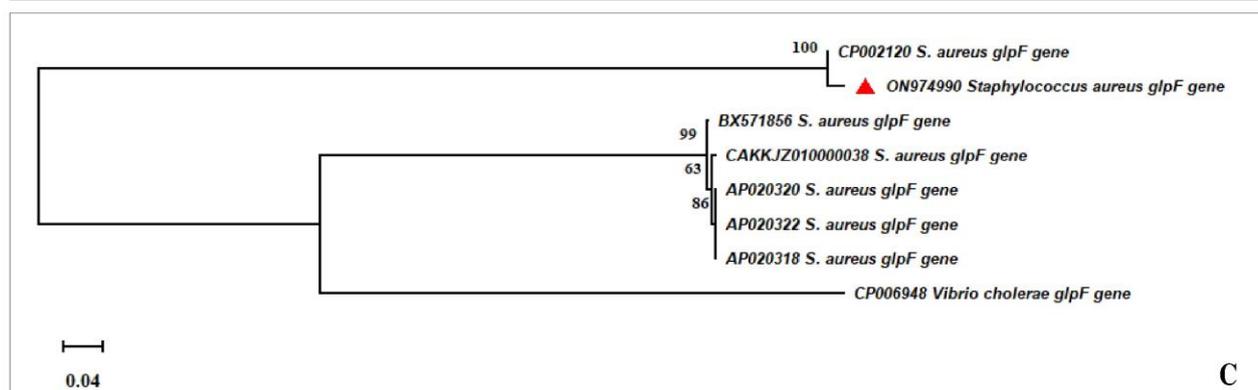
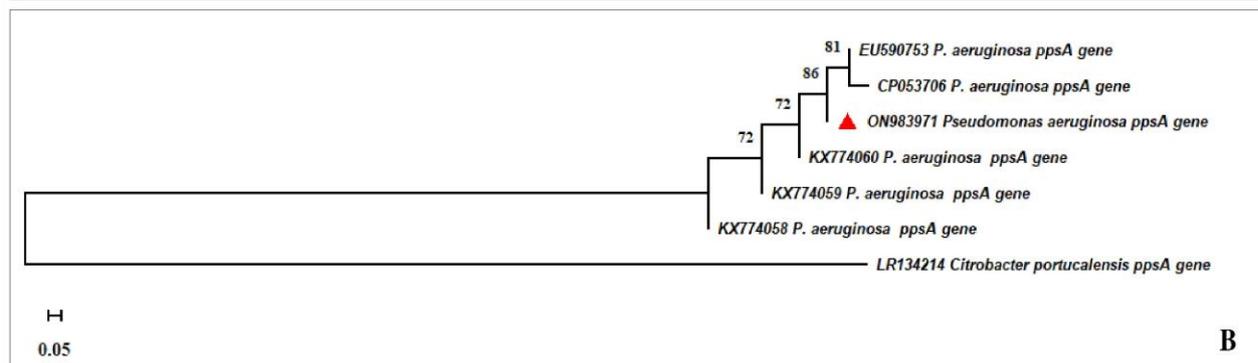
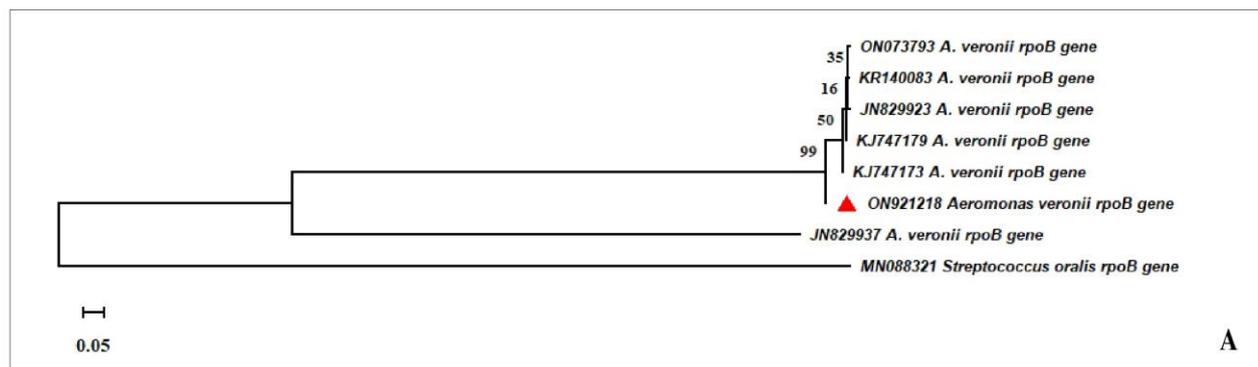


Figure S4. Photograph of agarose gel of PCR amplification of 16s rRNA. Lane 1–13 indicate amplicon of 16s rRNA gene with product size of 1450 bp; M: Molecular weight marker (500bp).



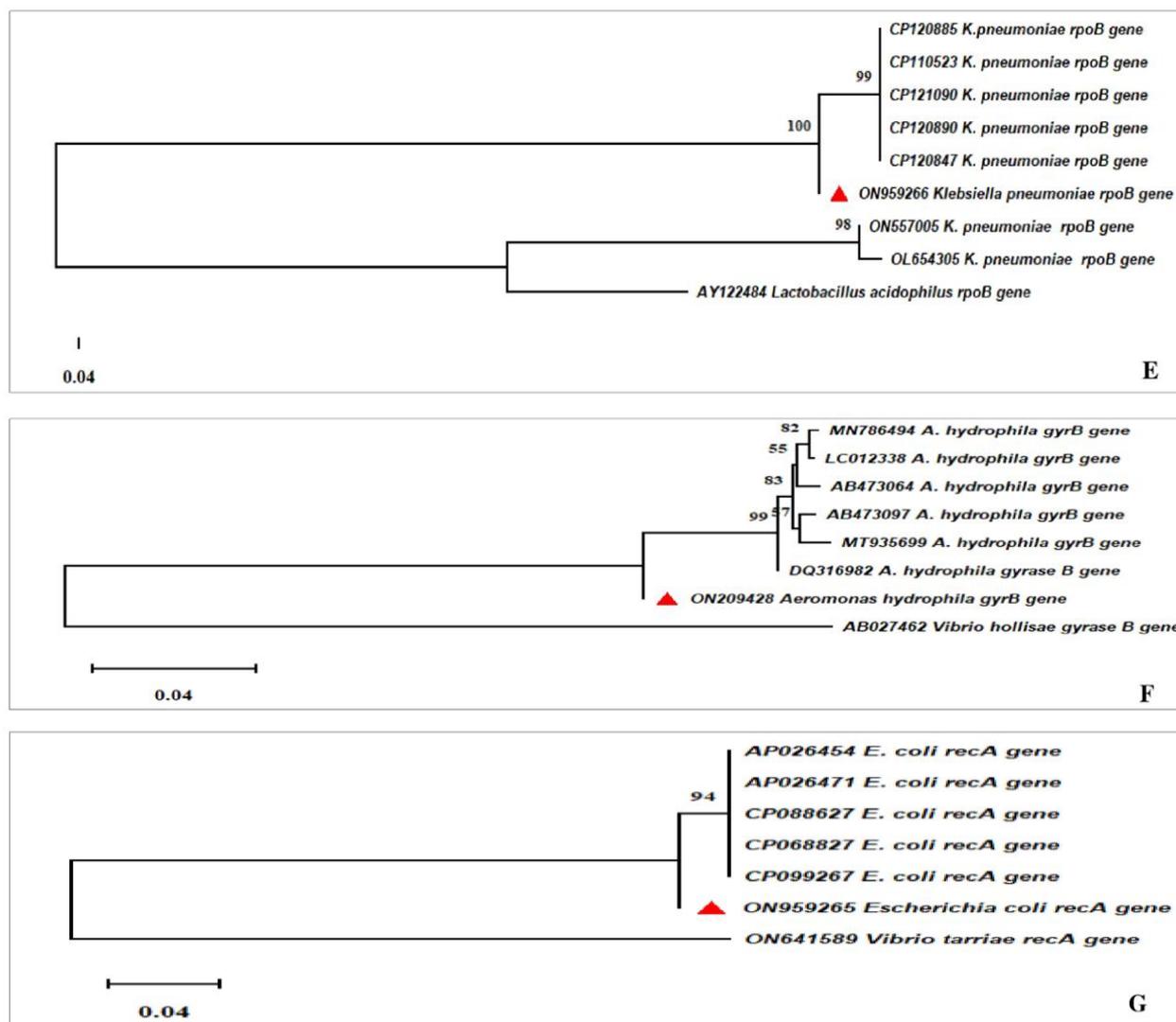


Figure S5. Phylogenetic analysis of isolated bacteria based on housekeeping gene sequences; **A:** rpoB gene sequences of *A. hydrophila*, **B:** ppsA gene sequence of *P. aeruginosa*, **C:** glpF gene sequence of *S. aureus*, **D:** gyd gene sequences of *E. faecalis*, **E:** rpoB gene sequences of *K. pneumoniae*, **F:** gyrB gene sequence of *Aeromonas hydrophila* and **G:** recA gene sequence of *Escherichia coli*. Phylogenetic analysis was inferred following Neighbor-Joining (NJ) method by MEGA 11 software. The numbers next to the branches indicate percentage values for 1000 bootstrap replicates. Red triangle represents our studied bacterial isolates obtained from diseased *L. rohita*.



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