Genomic and Therapeutic Insights into Multidrug-Resistant *Vibrio parahaemolyticus* BD001 Strain Isolated from Shrimp Acute Hepatopancreatic Necrosis Disease (AHPND): *In Vitro*, *In Vivo*, and *In Silico* Approaches

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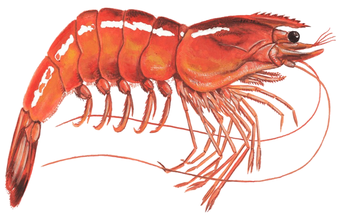
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Introduction

Shrimp aquaculture is crucial for global food security and economic growth. In 2020, production exceeded 4.8 million metric tons, valued at over **$40 billion USD** (Mandal and Singh, 2025).



The shrimp industry faces a **major threat** from Acute Hepatopancreatic Necrosis Disease (AHPND), caused by ***Vibrio parahaemolyticus***, with mortality rates reaching up to 100% within 20-30 days post-stocking (Li et al., 2017).

The World Health Organization (WHO) warns that antimicrobial resistance (AMR) could result in **10 million deaths annually** by 2050, surpassing cancer as a leading cause of death (Abadi et al., 2019).

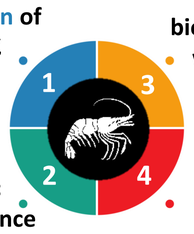
The overuse of antibiotics in shrimp farming has led to the emergence **of multidrug-resistant (MDR) strains** of *V. parahaemolyticus*, complicating both animal and human treatment efforts

Given the increasing failure of conventional antibiotics, attention has turned to phytochemicals—naturally derived plant compounds with broad-spectrum antimicrobial activity

Objectives

Isolation and identification of genomic features of *V. parahaemolyticus.*

Screening and evaluation of bioactive phytochemicals through in vitro and in vivo assay against *V. parahaemolyticus*.

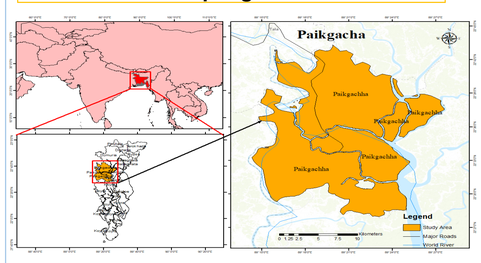


Detection of antibiotic resistant genes and virulence factors.

Proposal of novel, eco-friendly therapeutic alternatives to antibiotics for combating V. parahaemolyticus infections.

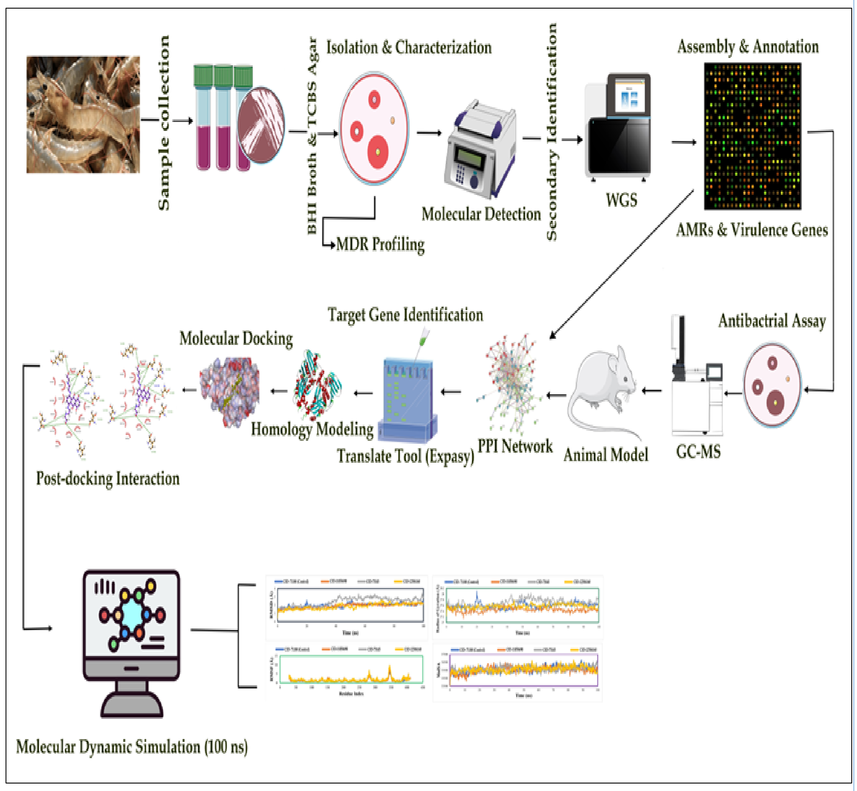
Methodology

Sampling Area



150 suspected samples from 20 sizeable farms located at Paikgachha upazila (22.5889°N 89.3361°E), Khulna, Bangladesh

Experimental design



- **Molecular detection and Antibiogram**: *Vibrio* spp. was initially identified using a universal primer (***groEL*** gene), followed by the detection *of V. parahaemolyticus* species using three species-specific primers (***groVp***, ***trh***, and ***tdh***) by multiplex PCR. Furthermore, **23 antibiotics** of 10 different classes were used for the antibiogram study.
- **Genome sequencing, assembly, and annotation of *V. parahaemolyticus***:

✓ The **highest antibiotic-resistant isolate** was selected for sequencing from **icddr,b** (Illumina MiSeq). Draft genomes assembled (SPAdes), annotated (**Prokka**), Subsystems via **RAST** and **PathogenFinder** for pathogen risk

✓ Bacteriocins via **BAGEL4**, Prophages/CRISPR via **PHASTEST/ICEFinder**; **VFDB** and **ResFinder** to detect virulence factor genes and AMR genes.

✓ **Roary tool** to perform a **pan-genome** analysis

- **Antibacterial activity of *Ixora chinensis* leaf extract**

✓ Leaf extracts prepared with 10% **DMSO** (10-150 mg/mL) and **saline** (80-100 mg/mL

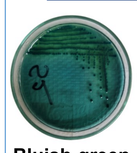
✓ Antibacterial activity tested by well diffusion method on MHA plates

✓ After confirming antibacterial activity **GC-MS** was performed commercially at **CSIRL Lab, JUST**.

Results

27 out of 150 field sample were positive for *Vibrio* genus and **12 out of 150** field sample were positive for ***V. parahaemolyticus***.

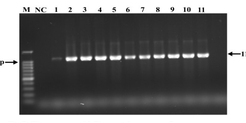
Cultural and staining properties with molecular detection



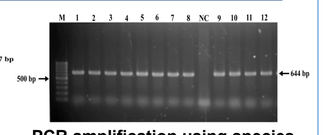
Bluish-green color colony



Staining property (rod-shaped)



PCR amplification of genus specific primers used to detect *Vibrio* genus. M=100 bp DNA ladder. Lane 1, 2, 3, 4, 5, 6, 7, 8,9, 10, 11: Suspected isolates, NC: Negative control.



PCR amplification using species specific primers of *groEL* gene to detect *V. parahaemolyticus*. M=100 bp DNA ladder, Lane 1, 2, 3, 4, 5, 6, 7, 8, 9,10, 11, 12: Isolated *Vibrio* spp., NC: Negative control

Antibiogram profile of *Vibrio parahaemolyticus*

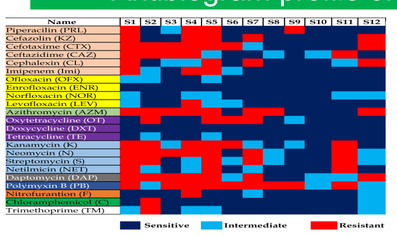


Fig: Heatmap of Antibiotic Resistance pattern

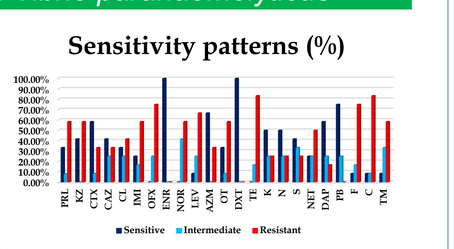


Fig: Summary of antibiotic sensitivity test

❑ **BD001 (S1)** exhibited the highest **resistance** to 13 antibiotics and **intermediate** to 4 others.

❑ **100%** sensitive to **enrofloxacin** and **doxycycline.**

Genomic features of *V. parahaemolyticus* BD001 Strain

Parameter	
Assembled genome size (Mb)	5.1
Guanine–cytosine %	45.21
Coverage (×)	149.33
Genome completeness (%)	98.74
Contamination (%)	3.51
Number of contigs	239
Largest contig (Mb)	4.8
Avg. Nucleotide Identity (%)	93
CDSs (with protein)	4578
tRNAs	138
tmRNAs	1
CRISPR arrays	3
Bacteriocin	1
Bacteriophage	54
Plasmid	1
Antibiotic resistant genes	6
Virulence factor genes	4

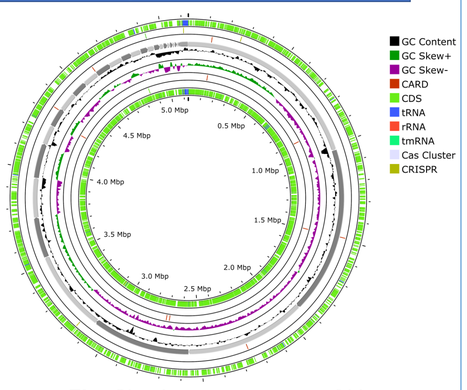


Fig: Circular genome map of *V. parahaemolyticus* BD001 strain

❑ Identified **379** functional pathways Primary involving:

❑ Carbohydrate metabolism (**11.6%**)

❑ Amino acid & derivatives (**17.4%**)

❑ Protein metabolism (**11.21%**)

❑ Cofactors, Vitamins, Prosthetic groups, and pigments (**9.69%**)

❑ Respiration (**4.4%**)

❑ Virulence, disease, and defense (**2.25%**)

❑ DNA and RNA metabolism (**3.6% & 2.5%**)

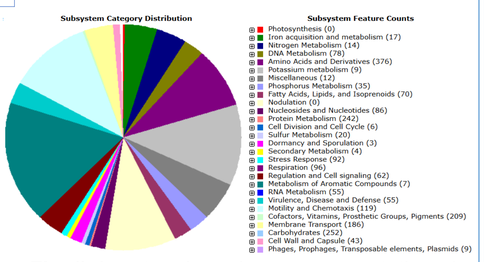


Fig: Subsystem-based genome annotation of BD001 strain

Pan genomic analysis of *V. parahaemolyticus*

❑ Selected **21** *V. parahaemolyticus* strains include BD001 from both clinical and environmental sources.

❑ Revealed **11,333 gene clusters**, comprising

- **3,155** core gene clusters
- **450** soft-core gene clusters
- **1,115** shell gene clusters
- **6,613** cloud gene clusters

❑ The core-to-pan genome ratio of **27.84%**

❑ BD001 strain closely related to **German strain**

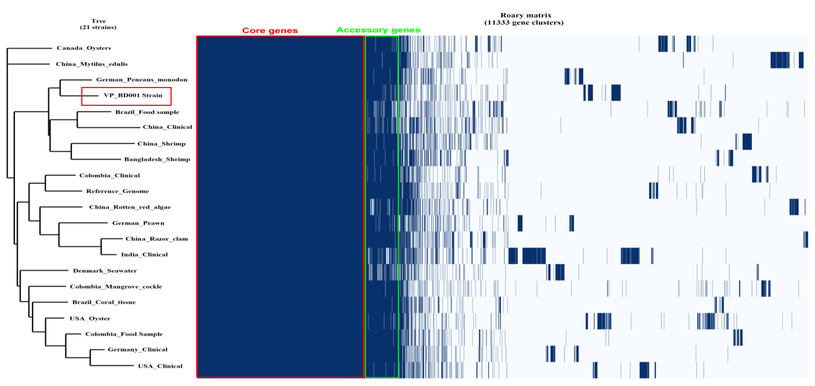


Fig: Pan-genome visualization by Roary of 21 valid genomes

Evaluation of GC-MS analysis and antimicrobial activity of *Ixora chinensis*

❑ In **GC-MS** analysis identified **41 distinct** compounds in **16 major** chemical classes such as

- Fatty acids **(29.27%)**
- Triterpenoids **(12.20%)**
- Sterols **(9.76%)**

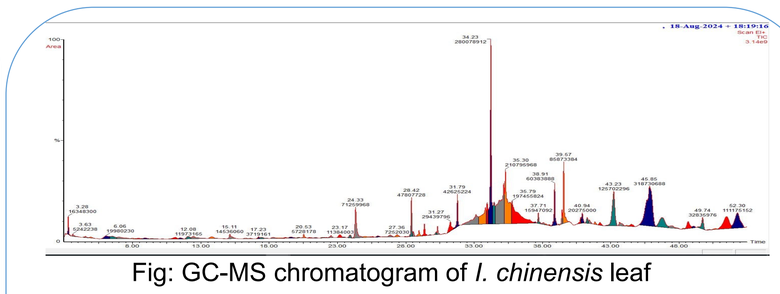


Fig: GC-MS chromatogram of *I. chinensis* leaf

Dose (mg/ml)	Zone of inhibition (mm)	Dose (mg/ml)	Zone of inhibition (mm)
Control (enrofloxacin)	25	100	19
10% DMSO	No zone	110	20
10	8	120	19
20	7	130	19
30	7	140	17
40	7	150	23
50	8	**Saline**	**No zone**
60	14	80-S	14
70	14	90-S	15
80	15	100-S	17
90	16		

Antimicrobial activity of *Ixora chinensis*

In vivo Study: pathogenesis of *V. parahaemolyticus* in mice

Liver functions

Groups	Functions		
	ALP	ALT	AST
Negative Control	224 ± 12.12	49.03 ± 3.48	116.17 ± 11.05
Positive Control	417.50 ± 3.82***	48.56 ± 1.77	112.11 ± 6.63
Plant Treatment (250 mg/kg)	226 ± 8.65***	57.58 ± 2.63*	135.18 ± 6.74**
Antibiotic Treatment (enrofloxacin)	279.70 ± 57.01	46.56 ± 3.14	139.75 ± 8.63*

Total viable count

Mice No.	Negative Control	Positive Control	Plant Treatment	Antibiotic Treatment
1	4.44	8.49	7.04	6.54
2	3.34	7.39	6.77	6.08
3	4.28	6.86	6.36	5.95
4	3.18	7.95	7.08	5.88
5	3.85	7.65	6.95	6.04
6	3.49	8.04	7.3	6.15
Mean ± SEM	3.76 ± 0.21	7.73 ± 0.23	6.92 ± 0.13	6.11 ± 0.09

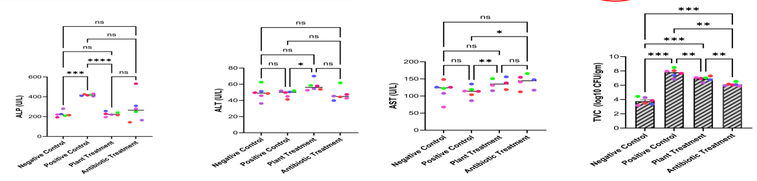


Fig: Efficacy of *I. chinensis* on *V. parahaemolyticus* infection *in vivo*. Liver functions: (A) ALP, (B) ALT, and (C) AST; (D) Total Viable Count (TVC)

In silico study: alternative antibiotics

❑ Z-score of **-8.84 and** Quality factor **94.3%**



Fig Homology-modeled 3D structures of BlaVeb-1 protein

❑ **Olean-12-ene**, **Beta-amyrone**, **Beta-amyrin** exhibits **highest** Pharmacophore fit scores – **79 %**

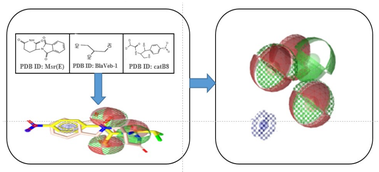


Fig: Shared feature pharmacophore (SFP) model of ARGs proteins

❑ **Docking score :** Control **(-8.8),** Olean-12-ene - **(-8.9),** Beta-amyrin - **(-8.6) and** Beta-amyrone - **(-8.5)**

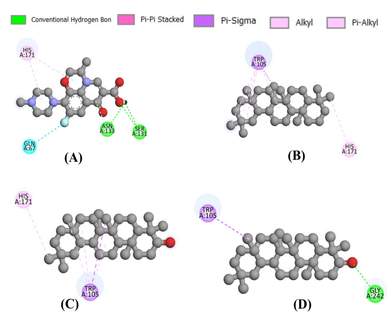


Fig: Post-docking interactions of **(A)** Control (ENR), **(B)** Olean-12-ene, **(C)** Beta-amyrin, and **(D)** Beta-amyrone with BlaVeb-1

Molecular dynamic simulation

Ligands	RMSD	RMSF	Rg	SASA	MolSA	Amount of H_2 Bond
Olean-12-ene	2.249	2.74	27.473	32150.518	35098.012	1292
Beta-amyrin	2.913	2.041	27.793	32574.259	36320.325	1349
Beta-amyrone	2.166	2.041	27.673	32214.021	35548.998	1316
Control (enrofloxacin)	2.229	2.449	27.494	31918.158	35171.871	1336

Summary of acceptable range

❑ **Root Mean Square Deviation (RMSD)**: **1 – 4.0 Å** (indicates accuracy of protein-ligand docking)

❑ **Root Mean Square Fluctuation (RMSF): < 3.5 Å** (reflects protein flexibility).

❑ **Radius of Gyration (Rg)**: **25 – 38 Å** (reflects protein compactness).

❑ **Solvent Accessible Surface Area (SASA)**: **> 25,000 Å²** (indicates protein-solvent interaction and exposure).

Conclusions

❑This study provides compelling evidence that the MDR strain BD001 poses a **growing risk to aquaculture and public health** due to its broad antibiotic resistance profile and genomic potential for horizontal gene transfer.

❑Isolation rate of *V. parahaemolyticus* **(8%)**

❑BD001 exhibited **resistance to 13 antibiotics** and **intermediate resistance to 4 others**.

❑Whole genome sequencing analysis, revealed a **5.1 Mb** genome size with **45.21%** GC content and **149.33x** coverage, encoding **4,578** CDSs, **138** tRNAs.

❑Identified **379** Functional pathways

❑Identified **6** AMRs, **4** Vir genes and **3** Crispr arrays.

❑***I. chinensis*** demonstrated **antibacterial activity** with inhibition zones up to **23 mm**.

❑**41 phytocompounds** identified by GC-MS

❑ ***In vivo*** treatment in mice reduced intestinal bacterial load after treatment **6.92 ± 0.13** and improved liver enzyme levels (**ALP: 226 ± 8.65, ALT: 57.58 ± 2.63, AST: 135.18 ± 6.74**).

❑These findings underscore the potential of *I. chinensis* phytocompounds highlight **olean-12-ene and beta-amyrone** are promising eco-friendly, bioactive agents for combating MDR *V. parahaemolyticus* in ***in silico*** study.

References

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Acknowledgments

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