PERIODICUM BIOLOGORUM
VOL. 126, No 1–2, 43–52, 2024
DOI: 10.18054/pb.v126i1-2.32185

UDC 57:61
CODEN PDBIAD
ISSN 0031-5362

Original research article

# Salinity-pathogenicity interaction of *Vibrio alginolyticus* in *Penaeus vannamei* shrimp

**ARUN SHARMA[1]***
**THONGAM IBEMCHA CHANU[1]**
**MURALIDHAR P. ANDE[2]**

[1] ICAR-Central Institute of Fisheries Education, Panch Marg, Versova, Mumbai, India

[2] ICAR-Central Institute of Fisheries Education, Kakinada Centre, Kakinada, Andhra Pradesh, India

***Correspondence:**
Arun Sharma
E-mail address: arunsharma@cife.edu.in

**Keywords:** *Penaeus vannamei*; *Vibrio alginolyticus*; mass mortality; virulence test; diseases

## Abstract

***Background and purpose:*** *The* Vibrio *spp. are indigenous bacteria in the marine environment and usually constitute the majority in normal microflora of farmed and wild penaeid shrimp. They are one of the most diverse and vital shrimp pathogens that cause heavy mortalities in aquaculture facilities worldwide. The present study aimed to isolate and characterize the causative agent of mass mortality in* Peneaus vannamei *collected from the shrimp farms of Amlapuram, Andhra Pradesh, India, and the salinity-pathogenicity interaction in the shrimp.*

***Materials and methods:*** *To isolate and characterize the pathogenic bacteria from the hepatopancreatic tissue of moribund,* P. vannamei *performed morphology, physiology, biochemical tests, and molecular analysis. Three virulence genes, namely ompK, toxR, and collagenase, were tested using PCR. In addition to that virulence gene study, bacterial pathogenicity with different salinity and antibacterial activity of the ethanol and the crude stem juice extract of* Tinospora cordifolia *against* Vibrio alginolyticus *were tested.*

***Results:*** *Bacterial isolates were phenotypically and genotypically identified as* Vibrio alginolyticus *strain AMLA01 (GenBank accession number: KY494863.1). The strain is positive for three virulence genes: ompK, toxR, and collagenase. The study concerning the interaction of bacterial pathogenicity with different salinity 0, 5, 10, 15, 20, and 25 ppt showed that 5 ppt to 20 ppt showed maximum resistance against bacterial infection in* P. vannamei *with higher CFU/shrimp value. In the case of 0 ppt and 25 ppt, the salinity CFU/shrimp value decreases, and bacterial pathogenicity increases. The antibacterial activity of both the ethanol and the crude stem juice extracts of* T. cordifolia*, exhibited antibacterial activity against* V. alginolyticus.

***Conclusions:*** *The cause for the mass mortality of juvenile shrimp* P. vannamei *in Amlapuram, Andhra Pradesh, India, was* Vibrio alginolyticus *strain AMLA01. Further work is necessary to isolate and purify the active constituents in stem extracts and examine the absorption pattern of the active ingredients of these plants, which will allow the scientific community to recommend their utilization as an accessible alternative to synthetic antibiotics.*

Received July 16, 2024
Revised October 3, 2024
Accepted October 4, 2024

## INTRODUCTION

*Penaeus vannamei*, Pacific whiteleg shrimp, is a worldwide suitable species for aquaculture development, with an annual production of 5.8 million tons *(1)*. The output is expected to increase at an annual

growth rate of 2.7% by 2030 *(2)*. The Indian shrimp market attained a volume of 0.93 million tons in 2023. The market is further expected to grow at a CAGR of 9.60% in the forecast period of 2024-2032, reaching a volume of approximately 2.12 million tons by 2032. Andhra Pradesh is the leading shrimp producer, accounting for over 50% of the total output *(3)*. The potential area available for brackish water aquaculture in Andhra Pradesh is about 150,000 ha, with a network of 172 brackish water bodies in 9 coastal districts *(4)*. This accounts for 12.6% of the total potential area in the country (1.2 million ha). Out of the total potential area in Andhra Pradesh, 84,951 ha (56.6%) has been developed for shrimp farming *(5)*. In India, as the area affected by inland saline water is increasing *P. vannamei* are being cultured in different salinities ranging from 0-35 ppt. In different salinity, the physiological response and the protective mechanism of *P. vannamei* against the pathogenic organism may vary. Similarly, the pathogenicity of the pathogen may also vary with salinity variation.

The higher growth of *P. vannamei* farming operations and infirmity in management practices become a possible cause of the incidence of diseases of both infectious and non-infectious origin *(6)*. The disease has become a severe restraint to aquaculture, leading to a subsequent increase in the cost of production year by year *(7)*. The *Vibrio* spp. are rich bacteria in the marine environment and usually constitute the majority in normal microflora of farmed and wild penaeid shrimp, and are one of the most diverse and important shrimp pathogens that cause heavy mortalities in aquaculture facilities worldwide *(8)*. Heretofore, from different penaeid shrimps, multiple pathogenic *Vibrio* strains *viz*. *Vibrio nigripulchritudo, V. alginolyticus*, *V. campbellii, V. parahaemolyticus*, *V. penaeicida*, *V. harveyi*, *V. splendidus* have been isolated and characterized *(9)*. In the Indian context, particularly in Andhra Pradesh districts, namely Nellore, Prakasam, Guntur, Krishna, West Godavari, East Godavari *P. vannamei* aquaculture industry faced a serious problem related to bacterial diseases *(10)*. Which results in this dumping of antibiotics and other chemicals in the shrimp culture ponds results in the emergence of antibiotic-resistant pathogens, and the rejection of shrimp consignment due to the detection of antibiotic residues in shrimp tissues by various importing countries has led to a need for alternatives to antibiotics in shrimp farming. Herbal drugs are primarily knuckled down as an alternative source against manifestations caused by various micro-organisms due to the abuse of antimicrobial drugs, pesticides, and disinfectants in aquaculture disease prevention and growth promotion, which has led to the progression of resistant strains of bacteria and questions of safeness *(11)*.

A *P. vannamei* farm in Amlapuram, Andhra Pradesh, India, succumbed to mass mortality, it was believed that the main pathogenic agent was from *Vibrio* sp., and suspected pathogenic bacteria *V. alginolyticus* was isolated from diseased shrimp. In a previous study, the *V. alginolyticus* infection was confirmed in *Lates calcarifer (12)*, tiger prawns (*Penaeus monodon*) *(13)*, white leg shrimp (*Litopenaeus vannamei*) *(14, 15)*. But, the pathogenicity study concerning different salinity, virulence genes, and herbal drug treatment is scanty and scattered. Therefore, in the present experiment, cause of illness of *P. vannamei* was thoroughly investigated. We studied the phenotypic characteristics of the isolated bacteria, their main biological characteristics, extracellular enzymes produced, the interaction of bacterial pathogenicity with different salinity, their 16S rRNA gene sequence, phylogenetic analysis, detected antibacterial sensitivity against herbal plant *Tinospora cordifolia extract*. This study also describes the pathogenicity of *V. alginolyticus* in terms of salinity variation and the presence of virulence genes, which can be a basis for the prevention and control of disease epidemiology.

## MATERIALS AND METHODS

### Experimental Animal

The moribund Pacific white shrimp *P. vannamei* samples were collected from the shrimp farms of Amalapuram, Andhra Pradesh, which were concerned with mass illness due to bacterial infection. The moribund shrimps were aseptically transported to the molecular biology laboratory of ICAR-CIFE, Kakinada centre, within two hours in sterile water using a plastic drum with an aeration facility at the water temperature of 28±3°C. The Physico-chemical parameters, including salinity, dissolved oxygen (DO), pH, alkalinity, hardness, and ammonia, were analyzed during sampling in the infected pond. The water quality parameters were analyzed following the standard protocols *(16)*. Animals were handled as per the Institute Animal Ethical Committee and Institutional Biosafety Committee (IBSC) guidelines.

### Bacterial isolation

The microbiological analysis was carried out following Sharma *et al. (17)*, by randomly picking ten numbers of shrimp from each pond, a total of 30 shrimp from three ponds, including moribund and normal. A known weight of tissue samples, *viz*. muscle and hepatopancreas of the infected shrimp, were collected aseptically by dissecting the shrimp. Tissues were homogenized in phosphate-buffered saline (PBS) and serially diluted in the same buffer for up to $10^{-7}$ dilutions. Triplicate samples were plated on thiosulfate citrate bile salt sucrose (TCBS) agar (Himedia, India) supplemented with 2.5% NaCl. The culture plates were incubated aerobically for 24 h at $37^0$C and further observed up to 48 h, 72 h, and 96 h at $35^0$C for any slow-growing bacterial colony and the color of the colonies formed.

## Phenotypic identification

The isolated bacterial microbiota was selected for phenotypic identification based on the higher virulence. The identification protocols include morphology, biochemical, and physiological confirmative tests based on the methodologies described by Bergey's Manual of Systematic Bacteriology *(18)* and the HIIMVIC biochemical test kit (Himedia India). Cell morphology was observed by using microscopy. The identification test includes gram staining, motility, colony color, the size of TCBS, oxidase, catalase, production of indole, $H_2S$, $NO_2$ production, hydrolysis of starch, growth in 0,1,3,5,10% NaCl, growth at $4^0$C, $30^0$C, $37^0$C, $40^0$C, Voges Proskauer, arginine dihydrolase, lysine decarboxylase, ONPG, citrate utilization, ornithine decarboxylase, inositol, mannitol, arabinose, sucrose, glucose, salicin, cellobiose, melibiose, sorbitol, etc. Growth at different NaCl concentrations (0%, 1%, 3%, 5%, and 10% (w/v)) was determined in peptone water (10 g peptone per liter). Each isolate was grown in tryptic soy broth (TSB, Himedia, India) supplemented with 2.5% NaCl at 35°C for 24 h, before being stored with 20% (v/v) sterile glycerol at -20 $^0$C.

## Screening for other important pathogens

Initially, the shrimps were screened for WSSV to confirm the presence of WSSV following a single-tube WSSV detection kit (Bangalore Genei, India). The genomic DNA was initially extracted from tissue samples following the manufacturer's protocol. Single-tube PCR amplification was performed from the genomic DNA template per the manufacturer's protocol *(19)*. Triplicate samples from each sampling area were analyzed for the diagnostic PCR. Apart from WSSV, Shrimps were also tested and screened for other pathogens, such as Infectious Myonecrosis Virus (IMNV) *(20)* and *Enterocytozoon hepatopenaei* (EHP) *(21)*.

## Genotypic identifications

The culture isolate was subjected to molecular analyses to compare and test the phenotypic determination. According to the manufacturer's protocol, the genomic DNA was extracted from the isolated bacteria using a Uniflex DNA isolation kit (Himedia, India). The 16S rRNA gene was amplified by PCR using universal primers *(22)* 27F, 5' AGA GTT TGA TCM TGG CTC AG 3' and 1492R, 5' TAC GGY TAC CTT GTT ACG ACT T 3'. The nucleotides of the 16S rRNA sequence were matched with the other microbes in the National Centre for Biotechnology Information (NCBI) database using the program Basic Local Alignment Search Tool (BLAST). The evolutionary history was inferred using the Neighbor-Joining method. The evolutionary distances were computed using the Maximum Composite Likelihood method. The analysis involved 11 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1353 positions in the final dataset. Evolutionary analyses were conducted using MEGA7 software.

## Virulence gene assay

The genomic DNA was extracted from the pure cultures of the isolated bacteria using a uniflex DNA isolation kit following the manufacturer's instructions (Himedia, India). Three virulence factor genes encoding the outer membrane protein (ompK), toxR gene, collagenase gene were respectively amplified by PCR using specific gene primers as described in Table 1. PCR amplification was performed for collagenase in a final volume of 25 µL and *ompK* and *toxR* genes 50 µl using a Hi-PCR Kit (Himedia, India). For amplification of the collagenase gene *(23)*, the reaction mixtures consisted of 2x Taq PCR master mix 12.5 µL, 0.5 µL forward and 0.5 µL reverse primers, respectively, 9.5 µL $ddH_2O$ and 2 µL DNA as a template. The thermal cycling conditions were optimized as an initial denaturation at $95^0$C for 5 min followed by a total of 35 cycles of denaturation at $95^0$C for 30 sec, annealing at $57^0$C for 30 sec, and extension at $72^0$C for 50 sec. While for other genes, toxR gene *(24)* and outer membrane protein (ompK) *(25)* the PCR was carried out using 1 µl of DNA template, 25 µl of 2x Taq PCR master mix, 1µl of each forward and reverse primers and sterile distilled water. The thermal cycling conditions were optimized as an initial denaturation at $94^0$C for 4 min followed by a total of 35 cycles of denaturation at $94^0$C for 1 min, annealing at $54^0$C for 1 min, and extension at $72^0$C for 1 min. The final extension was done at $72^0$C for 2 min. A reagent blank except for template DNA, for which sterile distilled water was used. The PCR products

**Table 1.** *PCR primers, targets, amplicon sizes used in the study.*

| Name of gene | Primer sequence (5'-3') | Product size (bp) | Length (bp) |
|---|---|---|---|
| Collagenase | f:CGAGTACAGTCACTTGAAAGCC | 737 | 22 |
| | r:CACAACAGAACTCGCGTTACC | | 21 |
| ToxR | f:GATTAGGAAGCAACGAAAG | 658 | 19 |
| | r:GCAATCACTTCCACTGGTAAC | | 21 |
| Ompk | f:GGCGGTCGCTCTGGTATT | 319 | 18 |
| | r:TTGCCATCGTAAGTGCTGTA | | 20 |

were electrophoresed on 1.5% agarose gel stained with ethidium bromide (1 mg $mL^{-1}$) and visualized through ultraviolet trans-illumination. A 50-bp DNA ladder was used as the size standard.

## Pathogenicity study

To study the pathogenicity of the causative bacterial pathogen, bath challenges were made against healthy *P. vannamei* juveniles using the isolated bacteria from the infected shrimps *(17)*. Healthy juveniles (tested negative for WSSV, IMNV and EHP) weighing approximately 7 g were acclimatized for one week in 10 ppt seawater. Animals were fed twice daily (09:00 to 17:00) with a commercial feed. Shrimps were distributed in a 50 L FRP tank with 20 animals, each with constant aeration. The dominant isolates were incubated in TSB (Himedia, India) supplemented with 2.5% NaCl and incubated at 35°C for 18 h, allowing each to reach the late exponential growth phase. Shrimps in triplicates were challenged with 2.0x $10^7$, 2.0x$10^6$, 2.0x$10^5$, 2.0x$10^4$, 2.0x$10^3$, 2.0x$10^2$, 2.0x$10^1$ CFU $mL^{-1}$ concentration of dominants bacterial isolates. Controls were used with the same number of larvae in filtered seawater without bacterial inoculation. Dead animals were collected immediately after death, and a bacterial investigation was performed. Pathogenicity of the isolated strains was analyzed by studying the mortality rate of the larvae, and average values were taken to calculate percentage mortality *(9)*. The experiment was continued for 96 h.

## $LD_{50}$ value determination

The $LD_{50}$ tests, with batches of ten prawns per dose (in duplicate), were conducted by intramuscular (i.m.) injection of 0.1 ml bacterial suspension ($10^6$, $10^5$, $10^4$, $10^3$ CFU/ml) into the shrimp at the site between 4th and 5th abdominal segments *(26, 27)*. Deaths of the tested animals were recorded daily for 7 days post i.m. injection. Re-isolation and identification of the bacteria from hepatopancreas or hemolymph of moribund shrimp post-bacterial challenge was conducted with TSA and TCBS plates. The bacterial count was done twice on isolates selected for challenging bioassays in *P. vannamei*. Isolates were grown in TSB supplemented with 2.5% NaCl and incubated at 35°C for 18 h. Samples were centrifuged at 2400x *g* for 20 min, and the cell pellet was suspended in 1 mL of sterile saline solution (2.5% NaCl). The bacterial solution was adjusted spectrophotometrically to an optical density of 1 at 580 nm. The bacterial count was done using the serial dilution method using plates with TSA supplemented with 2.5% NaCl and incubated at 35°C for 24 h.

## Pathogenicity study in terms of salinity variation

A challenge study was conducted using the bath challenge method to determine the $LD_{50}$ of the isolated strain *(17)*. To study the pathogenicity, healthy juveniles weighing approximately 7 g were acclimatized for fifteen days in different 0, 5, 15, 20, 25 ppt saline water tanks. Shrimps in triplicates were challenged with 2.0x$10^7$, 2.0x$10^6$, 2.0x$10^5$, 2.0x$10^4$, 2.0x$10^3$, 2.0x$10^2$, 2.0x$10^1$ CFU $mL^{-1}$ concentration of dominants bacterial isolates with respect to each salinity. The assays were performed for 4 days. The experimental doses were done according to the previous bacterial count. Values of pH, salinity, dissolved oxygen, and temperature were monitored daily. During each bioassay, mortality was recorded two times a day, and dead shrimp were removed. During the assay period, no cleaning of the tanks was done, and the temperature was maintained between 28-30°C to promote vibrio infection.

## Preparation of extract and antibacterial sensitivity test

The plants of *Tinospora cordifolia* were collected from trees growing on the Balabhadrapuram farm, ICAR-CIFE, Kakinada Centre. The collected plant stems were thoroughly washed with water to remove dirt and made into small pieces using a cutter. They were then shade-dried and ground well using a mixer grinder and sieved. For solvent extraction, the sieved powder was soaked with equal ethanol (1:1) for 48 h with continuous shaking *(28)*. The slurry was then filtered and washed to remove non-soluble fractions. The extracts were then taken to dryness in front of the fan. The filtered substance was then centrifuged (20,000 g for 30 min). After the centrifugation, the extracts were condensed at $35^0$C to evaporate the solvent residue. These extracts were re-suspended in ethanol to yield 50, 100, 150, 200, 250 mg residue $mL^{-1}$ solvent. Another portion of the fresh stems was blended to get the juice of the stems and was mixed with distilled water to make different concentrations of 10, 25, 50, 75, and 100%. Similarly, a negative control disc was prepared with sterile distilled water. The antibacterial activity of the stem extracts was tested *in vitro* using a disc diffusion assay (29). The inoculum size of the bacterial culture was standardized (30). A single colony from the bacteria species was selected and suspended in sterile saline until turbidity was comparable or adjusted to 0.5 McFarland units. Prepared 1000 mL of Mueller-Hinton agar with 3% NaCl and TCBS agar as per the manufacturer's instruction. The final inoculum size was standardized to $10^5$ CFU $mL^{-1}$ with the help of a turbidimeter. A diluted (0.2 mL) bacterial culture was poured into sterile 10 cm Petri plates containing 15 mL of Mueller-Hinton and TCBS agar medium and spread over agar plates using a sterile glass rod; 0.1 mL of each extract was applied per filter paper disc (Whatman no. 1, 5 mm diameter) and was allowed to dry before being placed on to the top layer of the agar plates. Similarly, two control discs (positive and negative) were prepared in addition to the extract-treated disc where ethanol was added in the positive control disc and sterile distilled water was used in the negative control disc. The plates were incubated at $37^0$C for 24 h. The

experiments were carried out in triplicate, and the antibacterial activity of the test material was observed through the zone of inhibition by measuring the diameter of the disc, including the diameter in mm. The inhibition diameter from the negative control was subtracted from that in the herbal extract test dishes, and the remaining area was calculated as the inhibition zone. The average diameter of the zone of inhibitions was recorded, and the results were expressed as mean ± standard deviation.

### Data analysis

One-way ANOVA was carried out using the SPSS statistics data package, and the mean was compared at a 0.05 % level.

## RESULTS AND DISCUSSION

### Pathology, Bacterial isolation, and Screening for other pathogens

The disease outbreak was in the semi-intensive *P. vannamei* farm of Amlapuram, Andra Pradesh, India. The average body weight of the affected shrimp ranged from 5.0 to 6.0 g on the day of sampling. The affected *P. vannamei* shrimps were pale HP with discoloration, erratic swimming, lethargy, loss of appetite, pale white muscles, pale red shells, and appendages breaking off the antenna. The range of Physico-chemical parameters of all the three pond water viz. temperature (28-30°C), pH (8.0- 8.5), dissolved oxygen (5.2-5.7 mg/L), alkalinity (170.2-180.5), salinity (12.2-14.5 ppt), hardness (5870-6000 ppm) and ammonia (1.02-1.4 ppm) were reported. After 24 h of incubation at $37^0$C under aerobic conditions, bacterial colonies appeared as short rods with a curved shapes, 2-5 mm diameter, opaque, yellow on TCBS agar. After 48 h incubation at $37^0$C, only one type of dominant colony was observed in all the plates. All the colonies were tested by Gram's staining method and viewed under a microscope and the same type of gram-negative curved-rod shape) bacteria were isolated from the diseased fish. PCR amplification was performed using the single tube WSSV detection kit (Bangalore Genei, India) from the genomic DNA of infected *P. vannamei*, which revealed that no positive PCR signals were observed in the tested shrimps. Shrimps were also found negative for other pathogens, such as Infectious Myonecrosis Virus (IMNV), *Enterocytozoon hepatopenaei* (EHP).

### Bacterial identification and virulence test

The phenotypic identification protocol revealed that the strain was positive for Oxidase test, Catalase test, production of Indole, $NO_2$, hydrolysis of starch, gelatin, Voges-Proskauer test, citrate utilization, Mannitol, Glucose, Sucrose, sorbitol, growth on 1%, 3%, 5% NaCl and $30^0$C, $37^0$C, $40^0$C temperature. The strain was negative for $H_2S$ production, Arginine dihydrolase, Oxidation of ONPG, Lysine decarboxylase, Inositol, Ornithine decarboxylase, Arabinose, Salicin, Cellobiose, Melibiose growth on 0%, 10% NaCl and at $4^0$C temperature (Table 2). Regarding genotypic identification, the NCBI blast search analysis confirmed that the isolated bacteria were *Vibrio alginolyticus* strain AMLA01. The PCR products of 16S rRNA were about 1500 bp after an Agarose gel electrophoresis was run, and it was demonstrated that the

**Table 2.** *Phenotypic characteristics of Vibrio alginolyticus strain AMLA01.*

| Characteristic | AMLA01 strain |
|---|---|
| Cell shape | Short rod with curve shaped |
| Size of colonies in TCBS | 3-5 mm diameter |
| Colony colour in TCBS agar | Yellow |
| Gram Staining | - |
| Motility | + |
| Oxidase | + |
| Catalase | + |
| Hydrolysis of | |
| Starch | + |
| Gelatin | + |
| Production of | |
| $H_2S$ | - |
| Indole | + |
| $NO_2$ | + |
| Growth in NaCl [%(w/v)] | |
| 0 | - |
| 1 | + |
| 3 | + |
| 5 | + |
| 10 | - |
| Growth at 4°C | - |
| Growth at 30°C | + |
| Growth at 37°C | + |
| Growth at 40°C | + |
| Vogesproskauer | + |
| Arginine dihydrolase | - |
| Lysine decarboxylase | - |
| ONPG | - |
| Citrate utilization | + |
| Ornithine decarboxylase | - |
| Inositol | - |
| Mannitol | + |
| Arabinose | - |
| Sucrose | + |
| Glucose | + |
| Salicin | - |
| Cellobiose | - |
| Sorbitol | + |
| Melibiose | - |

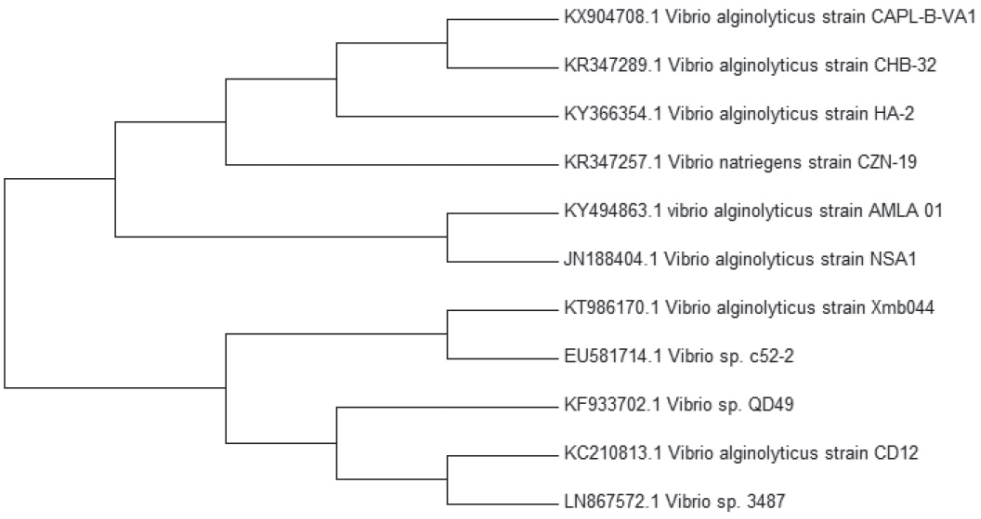


**Figure 1.** *Nucleotides homology and phylogenetic analysis of the microbe Vibrio alginolyticus based on 16S rRNA gene sequence data compare with other Vibrio sp. in the database.*

16S rRNA PCR products were 1353 bp by sequencing. Phylogenetic and evolutionary analysis of the 16S rRNA sequence revealed that the *V. alginolyticus* strain AMLA01 shared more than 99% similarities to other *V. alginolyticus* strains (Figure 1). The sequence was deposited in the NCBI database, and the strain name and gene bank accession number were *V. alginolyticus* strain AMLA01; KY494863.1. In the present study, the sequences of the 16S rRNA gene from the isolated bacterial strain *V. alginolyticus* strain AMLA01 showed high similarity with other *V. alginolyticus* strains in the GenBank database (NCBI) confirming *V. alginolyticus* strain AMLA01 was the responsible pathogen for mass mortality in *P. vannamei* samples collected from the shrimp farms of Amlapuram East Godavari district in Andhra Pradesh, India. Similarly, the *V. alginolyticus* infection was confirmed in *Lates calcarifer* cultured in open sea cages by biochemical and molecular methods *(12)*. *Vibrio alginolyticus* were also characterized and identified from tiger prawns (*Penaeus monodon*) *(13)*. In Malaysia, *Vibrio alginolyticus was also isolated from farmed white leg shrimp (Litopenaeus vannamei) (14)*. In Taiwanese culture ponds, *Vibrio alginolyticus* was isolated from diseased *L. vannamei (15)*. This result also signifies the fact that vibriosis is a major disease problem in shrimp aquaculture and vibriosis due to *V. alginolyticus* can devastate an entire shrimp farm causing high mortality and severe economic loss as it can attack various aquatic animals, such as tiger shrimp, the larvae of the Catarina scallop, gilt-head sea bream, giant freshwater prawn, mantis shrimp, diseased seaweed, *Gracilaria changii (14)*.

*Vibrios* are important bacterial pathogens for animals reared in aquaculture *(31)*, and several virulence factors are involved, *viz*. hemolysins and cytotoxins secretion, exotoxin production, etc., in the potential pathogenic capacity of *Vibrio* species *(32)*. In the present study, the specific virulence gene outer membrane protein (ompK), toxR gene, and collagenase gene fragments were obtained with the *V. alginolyticus* strain AMLA01 using a pair of ompK specific primers (319 bp region) and toxR gene-specific primers (658 bp region), collagenase gene (737 bp region), respectively (Figure 2). The presence of these genes justifies the fact that *V. alginolyticus* is a reservoir of potential virulence genes in the aquatic environment *(33)*.

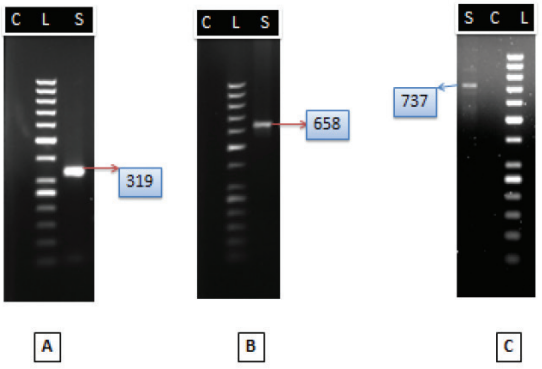


**Figure 2.** *(**A**): Lane-S: amplification of the 319 bp region of the ompK gene from the template DNA, Lane-C: negative control and Lane-L: 50 bp DNA ladder. (**B**): Lane-S: amplification of the 658 bp region of the toxR gene from the template DNA, Lane-C: negative control and Lane-L: 50 bp DNA ladder. (**C**) Lane-S: amplification of the 737bp region of the collagenase gene from the template DNA, Lane-C: negative control and Lane-L: 50 bp DNA ladder.*

Zulkifli *et al. (34) toxR* functions as a regulator for the expression of the virulence factor genes in other species of *Vibrio* such as *V. parahaemolyticus*. In the present study, isolates gave the amplification product for the toxR gene,

**Table 3.** *$LD_{50}$ value in bath and challenged study (n=10).*

| Isolate | Methods | Percentage of mortalities | | | | | | $LD_{50}$ value |
|---|---|---|---|---|---|---|---|---|
| | | Challenge doses (CFU/shrimp) | | | | | | |
| | | $10^2$ | $10^3$ | $10^4$ | $10^5$ | $10^6$ | *P. value* | |
| *V. alginolyticus* | Intramuscular injection | 20±0.176[a] | 50±0.915[b] | 80±1.23[c] | 100±1.48[d] | 100±0.37[d] | 0.012 | 3.161 x $10^3$ |
| | Bath challenge | 0.0±0.00 | 20±0.11[a] | 50±0.67[b] | 100±0.66[c] | 100±2.56[c] | 0.033 | 3.260 x $10^4$ |

Data are presented as mean ± SE and different superscripts a,b,c,d indicates significant difference ($p<0.05$) between the different CFU/shrimp. Total number of 3 replicates for each concentration.

which suggested that the toxin can regulate the expression of *ompK*. Thus, the role of the *ompK* gene in adaptation to the external environment changes can be related to *toxR* regulation. Collagenase plays a role in wound infection as the enzyme is capable of fastening the bacterial dissemination through the protein components of the extracellular matrix and causing hemorrhagic tissue damage through the destruction of the basement membrane by digestion of type IV collagen *(35)*. ompK followed by *toxR* and *collagenase* genes was isolated from diseased shrimp *Penaeus vannamei,* which indicated that this virulent factor could harm the shrimp.

## Pathogenicity study

The results of the challenge experiment for *P. vannamei* juveniles are given in Table 3. In the bath challenge, the $10^6$ CFU $mL^{-1}$ concentration and $10^5$ CFU $mL^{-1}$ concentration isolated strains caused 100% mortality of juveniles within 24 h of exposure. The $10^4$ CFU $mL^{-1}$ concentration caused 50% mortality of juveniles within 48 h of exposure. In the $10^3$ CFU $mL^{-1}$ concentrations, a lower mortality of 20% was observed. In the case of intramuscular injection, the $10^6$ CFU $mL^{-1}$ concentrations and $10^5$ CFU $mL^{-1}$ concentration isolated strains caused 100% mortality of juveniles within 24 h of exposure. The $10^4$ CFU $mL^{-1}$ concentrations caused 80% mortality of juveniles within 48 h of exposure. In the $10^3$ CFU $mL^{-1}$ concentrations, 50% mortality was observed. The bacterial isolates challenged against the shrimp could effectively kill the tested shrimp. The pathogenic capacity of the strain has been verified by fulfillment of Koch's postulates. The $LD_{50}$ values of *V. alginolyticus* strain AMLA01 for *P. vannamei* are 3.161 x $10^3$ CFU/shrimp ($p<0.05$) (injection method) and 3.260 x $10^4$ CFU/shrimp ($p<0.05$) (bath challenge method). Similarly, in Asian seabass, The $LD_{50}$ of *V. alginolyticus* was found to be 103.2 CFU/g fish when injected intramuscularly *(12)*. In *P. monodon* $LD_{50}$, the values were 1.13x $10^5$ CFU/g prawn body weight with intramuscular (i.m.) injection *(36)*. The calculated 96 h $LD_{50}$ dose of the isolated strain of *P. vannamei* was 3.0x$10^5$ colony forming units (CFU)/ shrimp injected into the ventral sinus of the cephalothorax *(15)*.

A study on the interaction of bacterial pathogenicity with different salinity showed that 5 ppt to 20 ppt showed maximum resistance against bacterial infection in *P. vannamei* with higher CFU/shrimp value. Whereas in the case of 0 ppt and 25 ppt, salinity CFU/shrimp value decreased and bacterial pathogenicity increased (Figure 3). This result justified the fact that Vibrios are halophytic bacteria, meaning they grow well in high-salinity aquatic environments, and their growth is inhibited when they are exposed to low-salinity water *(37)*. However, the exception in 0 ppt salinity where bacterial pathogenicity increases may be because, in 0 ppt, *P. vannamei* are not getting their minimum requirement for optimum growth as it can grow successfully at 5 to 35 ppt *(38)*. The possible biological mechanism of salinity stress and shrimp disease susceptibility may be explained in two ways: one way is to weaken the immune system of shrimp, making them more susceptible to pathogen infection; the other way is to cause dysbiosis in the gut microbiota, making it more susceptible to the invasion of pathogens by promoting the colonization of pathogenic bacteria *(39)*. In the present study, the 0 ppt salinity might have disrupted the stability of the diversity of shrimp gut microbiota and led to a shift in composition towards the dominance of opportunistic pathogens, corresponding to the high infection level of pathogenic bacteria *(39)*. In the case of 25 ppt salinity, bacterial pathogenicity increases may be because, even though the Pacific white shrimp has a wide range of salinity tolerance, the optimum salinity is around 20 ppt,

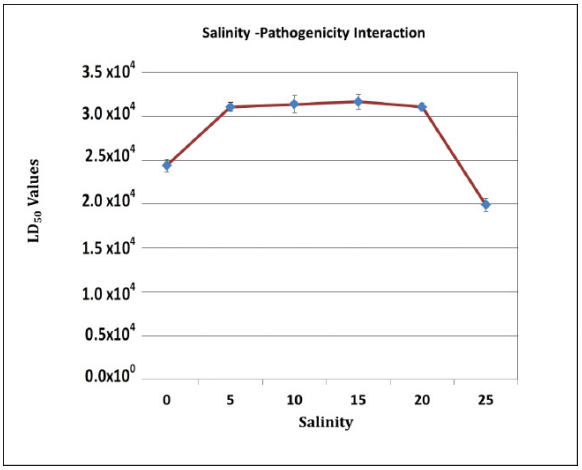


**Figure 3.** *Salinity pathogenicity interaction of V. alginolyticus.*

so maybe in 25 ppt salinity shrimp has invested additional energy by altering various enzymes and transporter proteins, which may increase their susceptibility to other stressors and diseases *(40, 41)*. Further study may be conducted regarding the increased pathogenicity of the bacteria at 0 ppt in terms of biodiversity and toxic gene expression.

### Antibacterial sensitivity test

The zones of inhibition in diameter (cm) recorded for ethanol crude stem juice are depicted in Table 4. The highest zone of inhibition was observed against *V. alginolyticus* with *Tinospora cordifolia* stem ethanol extract 14.6±0.43 cm ($p<0.05$) of the clear zone at 250 mg/ml concentration in TCBS agar followed by 13.8±0.43 cm ($p<0.05$) of the clear zone at 200 mg/ml concentration in Mueller-Hinton agar were observed. In the case of crude stem juice, 10.3±0.23cm ($p<0.05$) of the clear zone at 75 % concentration in Mueller-Hinton agar and 10.2±0.25cm ($p<0.05$) of the clear zone at 100 % concentration in TCBS agar were observed. The antibacterial sensitivity test revealed that the ethanol extracts and the crude stem juice exhibited antibacterial activity against *V. alginolyticus* but the more significant activity resides in plants' ethanol stem extracts than crude stem juice. This may *be* due to the chemical constituents responsible for the antibacterial activity being more soluble in ethanol extracts. Similarly, the ethanol extract also demonstrated significant antibacterial activity against *Escherichia coli, Proteus vulgaris, Enterobacter faecalis, Salmonella typhi, Staphylococcus aureus,* and *Serratia marcesenses* tested bacteria *(42)*. It can be interpreted that the antibacterial activity against micro-organisms is due to any one or more alkaloids of the plants *(30)*.

**Table 4.** *Antimicrobial activity of crude stem juice and ethanol extract of Tinospora cordifolia.*

| Test organism | Material | Inhibition zone (mm) | |
|---|---|---|---|
| | Ethanol extract (mg residue/ml solvent) | Mueller--Hinton agar | TCBS agar |
| | 50 | 6.7±0.12[a] | 7.1±0.32[a] |
| | 100 | 8.2±0.32[b] | 8.2±0.03[b] |
| | 150 | 8.1±0.21[b] | 13.2±0.53[c] |
| | 200 | 13.8±0.43[c] | 13.5±0.92[c] |
| | 250 | 13.4±0.23[c] | 14.6±0.43[d] |
| | -ve control | 0±0.00 | 0±0.00 |
| | +ve control | 8.9±0.02[b] | 8.3±0.02[b] |
| *Vibrio alginolyticus* | *P value* | *0.002* | *<0.001* |
| | Crude stem juice (%) | | |
| | 10 | 6.7±0.02[a] | 6.7±0.20[a] |
| | 25 | 7.3±0.27[a] | 7.3±0.50[a] |
| | 50 | 7.2±0.56[a] | 7.1±0.25[a] |
| | 75 | 10.3±0.23[b] | 9.2±0.75[b] |
| | 100 | 10.2±0.34[b] | 10.2±0.25[c] |
| | -ve control | 0.0±0.00 | 0.0±0.00 |
| | *P value* | *0.027* | 0.036 |

Data are presented as mean ± SE and Different superscripts a,b,c,d indicates significant difference ($p<0.05$) between the different extract doses (mg residue/ml solvent). Total number of 3 replicates for each concentration.

## CONCLUSIONS

In conclusion, we explored the reason for the mass mortality of juvenile shrimp *P. vannamei* was *Vibrio alginolyticus* strain AMLA01 in Amlapuram, Andhra Pradesh, India, which caused 100% mortality during bath challenge in $10^6$ CFU $mL^{-1}$ and $10^5$ CFU $mL^{-1}$ concentrations in 24 h, were positive for three virulence gene ompK, toxR, and collagenase. The study concerning the interaction of bacterial pathogenicity with different salinity 0, 5, 10, 15, 20, and 25 ppt showed that 5 ppt to 20 ppt showed maximum resistance against bacterial infection in *P. vannamei* with higher CFU/shrimp value. Meanwhile, in the cases of 0 ppt and 25 ppt, salinity CFU/shrimp value decreases, and bacterial pathogenicity increases. The study revealed that the ethanol extracts and the crude stem juice exhibited antibacterial activity against the isolated bacteria. Further work is necessary to isolate and purify the active constituents in *Tinospora cordifolia* stem extracts and observe the absorption pattern of the active ingredients of this plant, which will allow the scientific community to recommend their utilization as an accessible alternative to synthetic antibiotics.

***Acknowledgments:*** *The authors are thankful to the Director, ICAR-Central Institute of Fisheries Education, Mumbai, for providing the facility for conducting this study.*

## REFERENCES

1. FAO 2022 The state of world fisheries and aquaculture 2022. Towards blue transformation. Rome: FAO; 2022. https://doi.org/10.4060/cc0461en
2. OECD/FAO. OECD-FAO agricultural outlook 2021-2030. Paris: OECD Publishing; 2021. https://doi.org/10.1787/19428846-en
3. https://www.expertmarketresearch.com/reports/india-shrimp-market Latest accessed on 20.09.2024.
4. AQUACULTURE AUTHORITY OF INDIA 2001 Shrimp Aquaculture and the Environment: An Environment Impact Assessment Report submitted to the Supreme Court of India. Aquaculture Authority, Government of India, p. 114.
5. MPEDA 2006 Action plan for development of export oriented aquaculture in maritime states of India. MPEDA, Cochin, pp. 72.
6. LIGHTNER DV, REDMAN RM 1992 Penaeid virus diseases of the shrimp culture industry of the Americas. In: Fast AW and Lester L J (eds.) Marine shrimp culture: Principles and Practices,

Elsevier, Amsterdam, 569-588. https://doi.org/10.1016/B978-0-444-88606-4.50032-1

7. KUMARAN T, CITARASU T 2016 Isolation and Characterization of Vibrio Species from Shrimp and Artemia Culture and Evaluation of the Potential Virulence Factor. Intell Prop R 4: 153. https://doi.org/10.4172/2375-4516.1000153
8. BONDAD-REANTASO MG, SUBASINGHE RP, ARTHUR JR, OGAWA K, CHINABUT S, ADLARD R, TAN Z, SHARIFF M 2005 Disease and health management in Asian aquaculture. Veterinary Parasitology 132: 249-272. https://doi.org/10.1016/j.vetpar.2005.07.005
9. ZHANG XJ, YAN BL, BAI XS, BI K R, GAO H, QIN GM 2014 Isolation and characterization of Vibrio parahaemolyticus and Vibrio rotiferianus associated with mass mortality of Chinese shrimp (Fenneropenaeus chinensis). J Shellfish Res 33(1): 61-68. https://doi.org/10.2983/035.033.0108
10. VELMURUGAN S, PALANIKUMAR P, VELAYUTHANI P, DONIO MBS, BABU M. M, LELIN C, SUDHAKAR S, CITARASU T 2015 Bacterial White Patch Disease caused by Bacillus cereus, a new emerging disease in semi intensive culture of Litopenaeus vannamei. Aquaculture 444(1): 49-54. https://doi.org/10.1016/j.aquaculture.2015.03.017
11. ESIOBU N, ARMENTA L, IKE J 2002 Antibiotic Resistance in Soil and Water Environments. Int J Environ Health Res 12:133-144. https://doi.org/10.1080/09603120220129292
12. SHARMA SRK, RATHORE G, VERMA DK, SADHU N, PHILIPOSE KK 2013 Vibrio alginolyticus infection in Asian seabass (Lates calcarifer, Bloch) reared in open sea floating cages in India. Aquaculture Research 44:86-92. https://doi.org/10.1111/j.1365-2109.2011.03013.x
13. LEE KK, YU SR, CHEN FR, YANG TI, LIU PC 1996 Virulence of Vibrio alginolyticus isolated from diseased tiger prawn, Penaeus monodon. Current Microbiology 32: 229-231. https://doi.org/10.1007/s002849900041
14. LEE SW, WENDY W 2012 Characterization of Vibrio alginolyticus isolated from white leg shrimp (Litopenaeus vannamei) with emphasis on its antibiogram and heavy metal resistance pattern. Vet. Arhiv 82:221-227.
15. LIU CH, CHENG W, HSU JP, CHEN JC 2004 Vibrio alginolyticus infection in the white shrimp Litopenaeus vannamei confirmed by polymerase chain reaction and 16S rDNA sequencing. Dis Aquat Org 61:169-174. https://doi.org/10.3354/dao061169
16. APHA 1998 Standard methods for the examination of water and waste water. 20th Ed., American public health Association, Washington.
17. SHARMA A, CHANU TI, ANDE MP 2022 Detection and pathogenicity of Vibrio parahaemolyticus strains in Penaeus vannamei. Periodicum Biologorum 124(1-2):11-19. https://doi.org/10.18054/pb.v124i1-2.20578
18. VOS P, GARRITY G, JONES D, KRIEG NR, LUDWIG W, RAINEY FA, SCHLEIFER K, WHITEMAN W 2009 Bergey's Manual of Systematic Bacteriology: Volume 3: The Firmicutes (Bergey's Manual of Systematic Bacteriology, 3rd Edition, Springer-Verlag.
19. HAMEED SS, PARAMESWARAN V, SYEDMUSTHAQ S, SUDHAKARAN R, BALASUBRAMANIAN G, YOGANANDHAN K 2005 A simple PCR procedure to detect white spot syndrome virus (WSSV) of shrimp, Penaeus monodon (Fabricious). Aquaculture International, 13:441-450. https://doi.org/10.1007/s10499-005-7898-4
20. ANDRADE TPD, SRISUVAN T, TANG KFJ, LIGHTNER DV 2007 Real-time reverse transcription polymerase chain reaction assay using TaqMan probe for detection and quantification of infectious myonecrosis virus (IMNV). Aquaculture 264: 9-15. https://doi.org/10.1016/j.aquaculture.2006.11.030
21. TANG KFJ, PANTOJA CR, REDMAN RM, HAN JE, TRAN LH, LIGHTNER DV 2015 Development of in situ hybridization and PCR assays for the detection of Enterocytozoon hepatopenaei (EHP), a microsporidian parasite infecting penaeid shrimp. J. Invertebr. Pathol., 130: 37-41. https://doi.org/10.1016/j.jip.2015.06.009
22. LANE DJ, PACE B, OLSEN GJ, STAHL DA, SOGIN ML, PACE NR 1985 Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. Proc Natl Acad Sci- U S A 82: 6955-6959. https://doi.org/10.1073/pnas.82.20.6955
23. DI PINTO A, CICCARESE G, TANTILLO G, CATALANO D, FORTE VT 2005 A Collagenase-Targeted Multiplex PCR assay for identification of Vibrio alginolyticus, Vibrio cholerae, and Vibrio parahaemolyticus. J Food Prot 68(1):150-153. https://doi.org/10.4315/0362-028X-68.1.150
24. XIE ZY, HU CQ, CHEN C, ZHANG LP, REN CH 2005 Investigation of seven Vibrio virulence genes among Vibrio alginolyticus and Vibrio parahaemolyticus strains from the coastal mariculture systems in Guangdong, China. Lett. Appl. Microbiol 41(2):202-207. https://doi.org/10.1111/j.1472-765X.2005.01688.x
25. CAI S, LU Y, WU Z, JIAN J, HUANG Y 2009 A Novel Multiplex PCR Method for Detecting Virulent Strains of Vibrio Alginolyticus. Aquaculture Research 41(1):27-34. https://doi.org/10.1111/j.1365-2109.2009.02298.x
26. TREVORS JV, LUSTY CW 1985 A basic microcomputer program for calculating LD50 values. Water Air Soil Pollut 24:431-442. https://doi.org/10.1007/BF00282493
27. VERA P, NAVAS JI, QUINTERO MC 1992 Experimental study of the virulence of three species of Vibrio bacteria in Penaeus japonicus (Bate 1881) juveniles. Aquaculture 107:119-123. https://doi.org/10.1016/0044-8486(92)90055-P
28. ELOFF JN 1998 Which extractant should be used for the screening and isolation of antimicrobial components from plants? J Ethnopharmacol 60(1): 1-8. https://doi.org/10.1016/S0378-8741(97)00123-2
29. RASOANAIVO, RATSIMAMANGA-URVERY 1993 Biological evaluation of plants with reference to the Malagasy flora Monograph for the IFS-NAPRECA workshop on Bio-assays Antananavivo, Madagsascar, 72-79.
30. SATHISH N, SINGHARI AK 2003 Antimicrobial activity of roots of Coculus hirsutus. Anc Sci Life XXII(3): 168-172.
31. AUSTIN B, AUSTIN DA 1999 Bacterial fish pathogens; disease of farmed wild fish (3rd edn) springer - verlog KG, Berlin, Germany.
32. ANTONIO L, MANZANARES EM, BORREGO J 1996 Virulence factors of Vibrio P1, the causative agent of brown ring disease in the Manila clam, Ruditapes philippinarum. Aquatic Living Resources 9:125-136. https://doi.org/10.1051/alr:1996016
33. MUSTAPHA S, MUSTAPHA EM, NOZHA C 2013 Vibrio Alginolyticus: An Emerging Pathogen of Foodborne Diseases. International Journal of Science and Technology 2 (4):302-309.
34. ZULKIFLI Y, ALITHEEN NB, SON R, YEAP SK, LESLEY MB, RAHA AR 2009 Identification of Vibrio Parahaemolyticus isolates by PCR targeted to the ToxR gene and detection of virulence genes. International Food Research 296: 289-296.
35. NAJWA MN, DANIEL AMD, AMIN MKA, EFFENDY AWM 2015 Detection of virulence genes in Vibrio alginolyticus isolated from Green Mussel, Perna viridis. Jurnal Teknologi (Sciences & Engineering) 77(25):19-23. https://doi.org/10.11113/jt.v77.6731
36. LEE KK, YU SR, CHEN FR, YANG TI, LIU PC 1996 Virulence of Vibrio alginolyticus isolated from diseased tiger prawn, Penaeus monodon. Curr Microbiol 32(4):229-31. https://doi.org/10.1007/s002849900041

**37.** https://www.aquaculturealliance.org/advocate/low-salinity-culture-water-controls-vibrios-in-shrimp-postlarvae Last accessed on 20.09.2024.

**38.** PONCE-PALAFOX J, MARTINEZ-PALACIOS CA, ROSS LG 1997 The effects of salinity and temperature on the growth and survival rates of juvenile white shrimp Penaeus vannamei (Boone) 1931. Aquaculture 157:107-115. https://doi.org/10.1016/S0044-8486(97)00148-8

**39.** CHANG YT, HUANG WT, WU PL, KUMAR R, WANG HC, LU HP 2024 Low salinity stress increases the risk of Vibrio parahaemolyticus infection and gut microbiota dysbiosis in Pacific white shrimp. BMC Microbiology 24:275. https://doi.org/10.1186/s12866-024-03407-0

**40.** LI E, CHEN L, ZENG C, CHEN X, YU N, LAI Q, QIN JG 2007 Growth, body composition, respiration and ambient ammonia nitrogen tolerance of the juvenile white shrimp, Litopenaeus vannamei, at different salinities. Aquaculture 265:385-390. https://doi.org/10.1016/j.aquaculture.2007.02.018

**41.** MILLARD RS, ELLIS RP, BATEMAN KS, BICKLEY LK, TYLER CR, VAN AERLE R, SANTOS EM 2021 How do abiotic environmental conditions influence shrimp susceptibility to disease? A critical analysis focussed on White Spot Disease. J Invertebr Pathol 186:107369. https://doi.org/10.1016/j.jip.2020.107369

**42.** JEYACHANDRAN R, XAVIER TF, ANAND SP 2003 Antibacterial activity of stem extracts of Tinospora cordifolia (Willd) Hook. f & Thomson. Anc Sci Life XXIII(1): 40-43.