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Original Research Article

Detection and pathogenicity of *Vibrio parahaemolyticus* strains in *Penaeus vannamei*

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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; and 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 concerned with mass mortality in* Peneaus vannamei *collected from the shrimp farms of Kakinada, Andhra Pradesh, India.*

Materials and methods: *To isolate and characterize the pathogenic bacteria from the hepatic pancreatic tissue of moribund,* P. vannamei *performed conventional culture tests, physical and biochemical tests, and molecular analysis. In addition to that virulence gene study, bacterial pathogenicity with different salinity and antibacterial activity of the stem extract of* Tinospora cordifolia *against* Vibrio parahaemolyticus *were tested.*

Results: *The phenotypic traits, 16s rRNA gene, and phylogenetic tree analysis showed that the isolated pathogen was* V. parahaemolyticus strain KKD 02. *A study on bacterial pathogenicity with different salinity 0, 5, 10, 15, 20, and 25 ppt showed that 5 ppt to 10 ppt was connected to the maximum resistance against bacterial infection in* P. vannamei *with higher CFU/shrimp value. Two genes encoding thermostable direct hemolysin (*tdh*) and the thermostable direct hemolysin-related hemolysin (*trh*) were present in the isolate. The antibacterial activity of the stem extract of* T. cordifolia, *tested against* V. parahaemolyticus, *revealed that both the ethanol extracts and the crude stem juice exhibited antibacterial activity against* V. parahaemolyticus.

Conclusion: *The cause for the mass mortality of juvenile shrimp* P. vannamei *in Kakinada, Andhra Pradesh, India, was* V. parahaemolyticus *strain KKD 02. Further work is necessary to isolate and purify the active constituents in* T. cordifolia *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.*

INTRODUCTION

Penaeus vannamei represents over 90% of total shrimp production globally and is the most commonly cultured white shrimp in Latin America and South-East Asian countries *(1).* Commercialization of the *P. vannamei* commonly known as Pacific white shrimp in India, has resulted in significant growth in the export earnings from the export of processed shrimps. The shrimp farmers attained tremendous success and

huge profits, turning the other farmer's concentration to culture this exotic species *(2).* Marine exports, which comprise frozen shrimps, fin-fish, cuttlefish, squid, dried items, and others, constituted 2.1% of the total Indian export earnings from goods (USD 274.6 billion) during the financial year 2017 *(3).* Among them, frozen shrimps rank first in the marine export basket with an export volume of 1.13 million tonnes valued at USD 5.78 billion. The frozen shrimp segment comprised a share of about 65% with a volume contribution of 38% from the mentioned export value (of which 76% was contributed by *P. vannamei*) (*3*). Among the states in India, Andhra Pradesh tops the chart with a share of around 65% of the total shrimp production in India. Andhra Pradesh has a total cultivable brackish water area of about 28,000 hectares, from which it has allocated 57% to shrimp farming with regard to the national area under cultivation at 14% *(3*). As the area affected by inland saline increases in India, *P. vannamei* is cultured in different salinities ranging from 0-35 ppt. Different salinity can cause variation in the physiological response and the protective mechanism of *P. vannamei* as well as the virulence and pathogenicity of the pathogen itself.

Excessive production of *P. vannamei* and inadequate farming management practices become possible causes of the incidence of diseases of both infectious and non-infectious origin *(4).* Diseases have become a limiting factor in the development of shrimp aquaculture, leading to a subsequent increase in the production cost year by year *(5).* The *Vibrio* spp. are indigenous bacteria in the marine environment and usually constitute the majority in normal microflora of farmed and wild penaeid shrimp. *Vibrio* spp. is one of the most diverse and important shrimp pathogens that cause heavy mortalities in aquaculture facilities worldwide *(6).* Heretofore, from different penaeid shrimp's multiple pathogenic vibrio strains namely *Vibrio nigripulchritudo, Vibrio alginolyticus*, *Vibrio campbellii, Vibrio parahaemolyticus*, *Vibrio penaeicida*, *Vibrio harveyi*, *Vibrio splendidus* have been isolated and characterized *(7).* In the Indian context, particularly in Andhra Pradesh districts: namely Nellore, Prakasam, Gundur, Krishna, West Godavari, and East Godavari *P. vannamei* aquaculture industry faced a serious problem related to bacterial diseases *(8).* Which caused the dumping of antibiotics and other chemicals in the shrimp culture ponds, resulting in antibiotic-resistant pathogens. Rejection of shrimp consignment due to detection of antibiotic residues in shrimp tissues by various importing countries has led to a need for alternatives to antibiotics in shrimp farming. Now-a-days, plants have been exploited as a powerful and potential source for medicinal drugs *(9).* Herbal medicines and phytonutrients or nutraceuticals continue to expand rapidly across the world with many people now resorting to these products for the treatment of various health challenges *(10).* The reason behind the paradigm shift in the aquaculture industry for the production of farm-raised fish and shrimps is the unregulated use of antibiotics which could pose human health and food safety concerns due to the presence of drug residues, even in very low concentrations, in the edible tissues of the treated animal *(11).*

In May 2017 at a *P. vannamei* farm in the Kakinada, Andhra Pradesh, India, the cultivated shrimp succumbed to mass illness. Initial analysis suggests that the causative agent may be *Vibrio* sp. and a pathogenic bacteria *V. parahaemolyticus* was isolated from diseased shrimp. In the previous study, *V. parahaemolyticus, (12), V. vulnificus*, *(13),* and *Bacillus cereus (14)* were reported in *L. vannamei* but the pathogenicity study with respect to different salinity, virulence gene, and herbal drug treatment is very much scanty and scattered. Therefore, in the present experiment, the cause of the 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, and phylogenetic analysis, and detected antibacterial sensitivity against herbal plant *Tinospora cordifolia* extract. This study also describes the pathogenicity of the *V. parahaemolyticus* in terms of salinity variation and presence of virulence gene which can be a basis for 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 Kakinada, Andhra Pradesh, where concerned to mass illness to bacterial infection. The moribund shrimp's average weight 5.96±0.15 (g) and length 7.82±0.07 (cm), were aseptically transported to the molecular biology laboratory of ICAR-CIFE, Kakinada centre within two hours in sterile water using plastic drum with aeration facility at the water temperature 28±2 °C.

Physicochemical parameters of water during disease outbreaks

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 *(15).*

Bacterial isolation

The microbiological analysis was carried out by randomly picking ten numbers of shrimp from each pond, a total of 30 moribund shrimp from three ponds. A known weight of muscle and hepatopancreas tissue samples of the infected shrimp have 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 vibrio selective thiosulfate citrate bile salt sucrose (TCBS) agar (Himedia, India) supplemented with 2.5% NaCl. The culture plates were incubated aerobically for 24 h at 35 ^{0}C and further observed up to 48 h, 72 h and 96 h at 35 ^{0}C for any slow-growing aerial colony. The initial isolation plates showed predominant green and fewer yellow colonies. Only green colonies were selected and streaked onto TCBS plates and incubated as above. 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 for confirming the presence of WSSV following single tube WSSV detection kit (Bangalore Genei, India). Genomic DNA was extracted from tissue samples following manufacturer's protocol. Single tube PCR amplification was performed from the genomic DNA template as per the manufacturer's protocol. 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) *(16), Enterocytozoon hepatopenaei* (EHP) (17).

Phenotypic identification

The bacterial isolates were selected for phenotypic identification based on the higher virulence (more severely diseased shrimp). The identification protocols Including biochemical and physiological confirmative tests based on the methodologies described by Bergey's Manual of Systematic Bacteriology *(18)* and HIIMVIC biochemical test kit (Himedia India). The identification test includes gram staining, motility, oxidase, catalase, urease, indole, H_2S production, inositol, Vogesproskauer, arginine, ONPG, citrate, ornithine, mannitol, arabinose, sucrose, glucose, salicin, cellobiose, colony color on TCBS, TCBS growth, growth on 0% NaCl, growth in 1% NaCl, growth on 3% NaCl, etc.

Molecular identification of isolates

The culture isolate was subjected to molecular analyses in order to compare and test the phenotypic determination. The genomic DNA was extracted from the isolated bacteria using a uniflex DNA isolation kit (Himedia, India) as per the manufacturer's protocol. The 16S rRNA gene was amplified by PCR using universal primers *(19)* 27F, 5' AGA GTT TGA TCM TGG CTC AG 3' and 1492R, 5' TAC GGY TAC CTT GTT ACG ACT T 3'. Partial 16S rRNA sequences were matched with the other sequences in the database of the National Centre for Biotechnology Information (NCBI) using the program Basic Local Alignment Search Tool (BLAST). The evolutionary history was inferred using the Neighbor-Joining method. The Phylogenetic tree was constructed using MEGA11 software (*20*). The evolutionary distances were computed using the Maximum Likelihood method and Tamura-Nei model. The analysis involved 12 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1542 positions in the final dataset.

Virulence gene assay

The genomic DNA was extracted from the pure bacterial cultures using a uniflex DNA isolation kit following the instructions of the manufacturer (Himedia, India). Two virulence factor genes encoding the thermostable direct hemolysin (*tdh*) and the thermostable direct hemolysin-related hemolysin (*trh*) were respectively amplified by PCR using specific *tdh* and *trh* gene primers *(21)*. PCR amplification of virulence genes was carried out in a reaction volume of 25 mL by using a Hi-PCR Kit (Himedia, India). The final concentrations in the PCR mixture were 2x Taq PCR MasterMix 12.5 µL, 0.5 µL forward, and 0.5 µL reverse primers, respectively, 10.5 µL ddH_2O and 1 µL DNA as 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 1 min, annealing at 62 ^{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 blanks except for template DNA for which sterile distilled water was used as control. The PCR products 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, bacterial count, pathogenicity study in terms of salinity variation

In order 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. Healthy juveniles (tested negative for WSSV, IMNV and EHP) weighing approximately 7g were acclimatized for one week in 10 ppt sea water. Animals were fed twice daily (09:00 to 17:00) with a commercial feed. Shrimps have then distributed in 50 L FRP tanks 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 $2.0x10^7$, $2.0x10^6$, $2.0x10^5$, $2.0x10^4$, $2.0x10^3$, $2.0x10^2$, $2.0x10^1$ CFU mL^{-1} concentration of dominant bacterial isolates. Controls were used with the same number of larvae in filtered seawater without any bacterial inocula-

tion. 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 for calculating percentage mortality *(7).* The experiment was continued for 96 h. 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 with the serial dilution method using plates with TS agar supplemented with 2.5% NaCl and incubated at 35 °C for 24 h.

A Challenge study was conducted in the bath challenge method to determine the LD_{50} of the isolated strain. In order to study the pathogenicity, healthy juveniles weighing approximately 7g were acclimatized for fifteen days in different 0, 5, 10, 15, 20, 25 ppt saline water tanks. Shrimps in triplicates were challenged with $2.0x10^7$, $2.0x10^6$, $2.0x10^5$, $2.0x10^4$, $2.0x10^3$, $2.0x10^2$, $2.0x10^1$ 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 made, and the temperature was maintained between 28-30°C to promote vibrio infection.

Plant material and Extraction

The plants of *Tinospora cordifolia* were collected from trees growing in 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 by a using mixer grinder and sieved. For solvent extraction, the sieved powder was soaked with an equal part of ethanol (1:1) for 48 h with continuous shaking *(22).* The slurry was then filtered and washed to remove non-soluble fractions. The filtered extracts were 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, and 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, the negative control disc was prepared with sterile distilled water.

Antibacterial sensitivity test

The antibacterial activity of the stem extracts was tested *in-vitro* using a disc diffusion assay *(23).* The inoculum size of the bacterial culture was standardized *(24).* 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 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 controls disc (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 in mm inclusive of the disc. 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.

Statistical analysis

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

RESULTS AND DISCUSSION

Presently, the disease outbreak was found in the semi-intensive *P. vannamei* farm of Kakinada, Andra Pradesh, India. The affected *P. vannamei* shrimps were pale HP with discoloration, loss of appetite, lethargy *(25),* erratic swimming, pale white muscles, pale red shell, and appendages, and breaking of antenna. The range of Physico-chemical parameters of all the three ponds water viz. temperature (28-30°C), pH (8.0-8.5), dissolved oxygen (5.2-5.7 mg L^{-1}), alkalinity (170.2-180.5 ppm), 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 35 ^{0}C under aerobic conditions, colonies that were round in shape, 2-3 mm in diameter, opaque, and green were selected and used in further analyses. Initially, the shrimps were screened for WSSV, Infectious Myonecrosis Virus (IMNV), *Enterocytozoon hepatopenaei* (EHP) but the results were negative for these pathogens.

The phenotypic identification protocol revealed that the suspected isolated microbiota was Gram-negative, curved-rod shape, motile bacteria. The strain was positive for the Oxidase test, Catalase test, Indole production, Inositol, citrate utilization, Ornithine decarboxylase, Mannitol, Arabinose, Glucose, TCBS growth, growth in 1% NaCl growth in 3% NaCl. The strain was negative for Urease, H_2S production, Voges-Proskauer test, Arginine dihydrolase, Oxidation of ONPG, Sucrose, Salicin, Cellobiose, and growth on 0% NaCl (Table 1). The PCR products of 16S rRNA were 1420 bp long and NCBI blast search analysis confirmed that the isolated bacteria were *Vibrio parahaemolyticus*. The sequence was deposited in the NCBI database, under the strain name and gene bank accession number *Vibrio parahaemolyticus* strain KKD 02, KY495224. Phylogenetic and evolutionary analysis of the 16S rRNA sequence revealed that the *Vibrio parahaemolyticus* strain KKD 02 shared 99% similarities with other *V. parahaemolyticus* strains (Figure 1). The obtained result suggests that *V. parahaemolyticus* strain KKD 02 was the responsible pathogen for mass mortality in *P. vannamei* samples collected from the shrimp farms of Kakinada, Andhra Pradesh, India. Similarly, in Chinese shrimp *Fenneropenaeus chinensis* and Pacific white shrimp *Litopenaeus vannamei, V. parahaemolyticus* infections were confirmed by biochemical and molecular studies *(7,26)*. This result also signifies the fact that vibriosis is a major disease problem in shrimp aquaculture, causing high mortality and severe economic loss in aquaculture particularly in confined heavily stocked, commercial systems *(5)* and highlights *V. parahaemolyticus* as one of the major species causing vibriosis in shrimp *(27)*.

Table 1. *Biochemical characteristics of Vibrio parahaemolyticus isolates.*

Sl. No.	Test	Results
1.	Gram Staining	-ve
2.	Motility	+ve
3.	Oxidase	+ve
4.	Catalase	+ve
5.	Urease	-ve
6.	Indole	+ve
7.	H_2S	-ve
8.	Inositol	+ve
9.	VP	-ve
10.	Arginine	-ve
11.	ONPG	-ve
12.	Citrate	+ve
13.	Ornithine	+ve
14.	Mannitol	+ve
15.	Arabinose	+ve
16.	Sucrose	-ve
17.	Glucose	+ve
18.	Salicin	-ve
19.	Cellobiose	-ve
20.	Colony color on TCBS	green
21.	TCBS growth	good
22.	Growth on 0% NaCl	-ve
23.	Growth in 1% NaCl	+ve
24.	Growth on 3% NaCl	+ve

Vibrios are important bacterial pathogens for animals reared in aquaculture *(28)* and several virulence factors involved in haemolysins and cytotoxins secretion, exotoxin production etc. build in the potential pathogenic capacity of *Vibrio* species *(29)*. In the present study, the fragments of specific virulence genes: thermostable direct hemolysin (*tdh*) and thermostable direct hemolysin-related hemolysin (*trh*) fragments were amplified from the obtained with the *V. parahaemolyticus* strain KKD 02 using the pair of *tdh* (245 bp region) and *trh* (410 bp region)

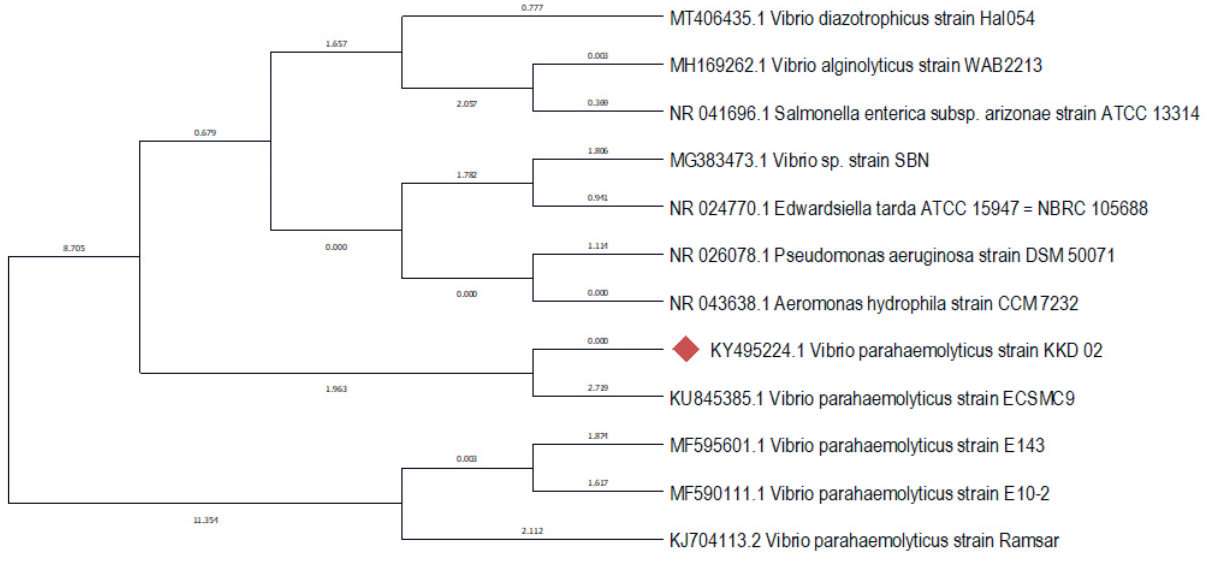

Figure 1. *Nucleotides homology and phylogenetic analysis of the microbe Vibrio parahaemolyticus based on 16S rRNA gene sequence data compare with other Vibrio spp. in the database.*

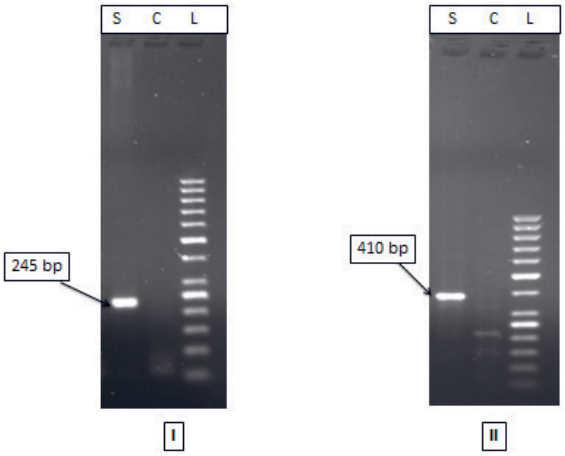

Figure 2. *(I): Lane-S: amplification of the 245 bp region of the tdh gene from the template DNA, Lane-C: negative control and Lane-L: 50 bp DNA ladder. (II): Lane-S: amplification of the 410 bp region of the trh gene from the template DNA, Lane-C: negative control and Lane-L: 50 bp DNA ladder.*

PCR specific primers (Figure 2). The presence of these two genes considered major virulence factors in V. *parahaemolyticus (30,31)* suggests the potential pathogenicity of the isolated strain. Similarly, *tdh+ V. parahaemolyticus* were reported in Alabama oysters *(32)* and *tdh* and *trh* virulent genes were detected in the strains of *V. parahaemolyticus* isolated from the shrimp *Penaeus monodon (33).* Consequently, *tdh* and *trh* positive strains of *V. parahaemolyticus* were isolated from Pristine Estuary *(21)* and from environmental samples *(34).*

The results of the bath challenge experiment for *P. vannamei* juveniles in the 10^7 CFU mL^{-1} concentration and 10^6 CFU mL^{-1} concentration, isolated strains caused 100% mortality of juveniles within 24 h of exposure. The 10^5 CFU mL^{-1} concentration caused 50% mortality of juveniles within 48 h of exposure. In the 10^4 CFU mL^{-1} concentrations, lower mortality of 15% was observed. The bacterial isolates challenged against the shrimp could effectively kill the tested shrimp (Figure 3). The pathogenic capacity of the strain has been verified by fulfillment of Koch's postulates. The pathogenicity study revealed that the bacterial microbiota from the three ponds was highly virulent and effectively killed the tested shrimp within ten days. Similarly, in *P. monodon* LD_{50} for *V. parahaemolyticus* was 10^5-10^6 CFU $shrimp^{-1}$ when injected IM for 7 days *(35)* and in. *L vannamei* a density of 10^4 CFU mL^{-1} caused 63% mortality at 104 post-infection (hpi) and below that

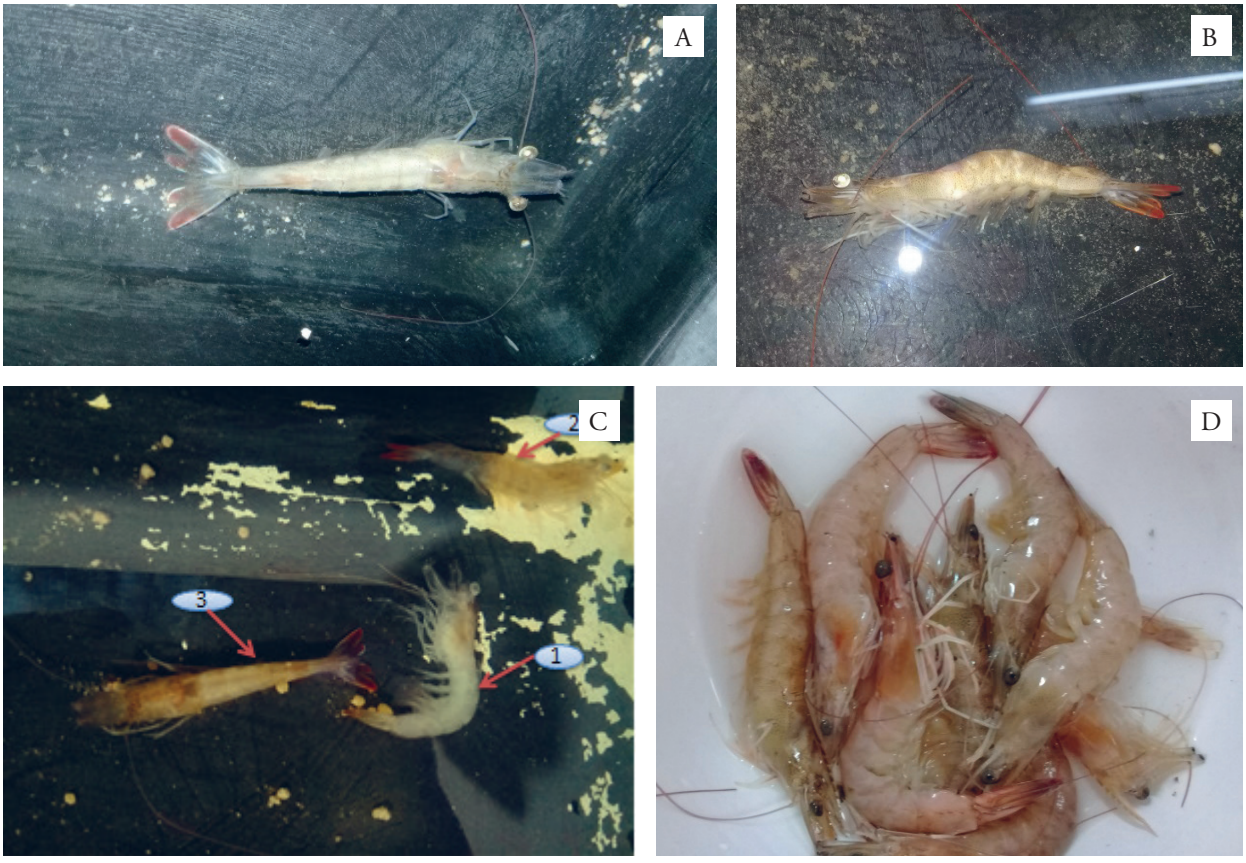

Figure 3. *Pathogenicity study of Penaeus vannamei against V. parahaemolyticus strain KKD 02. (A) Shrimp with major symptoms like pale HP with discoloration, pale white muscles, pale red terminal appendages (telson); (B) Moribund shrimp with major symptoms; (C) (1) dead shrimp, (2) moribund shrimp, (3) shrimp with all the major symptoms of infection; (D) Dead shrimps.*

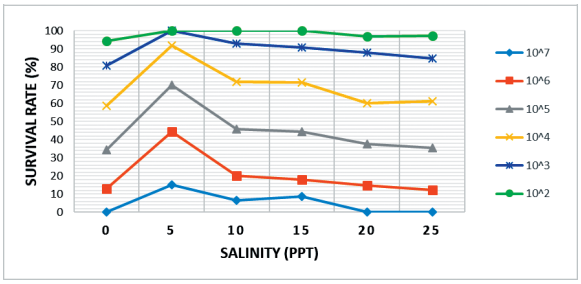

Figure 4. *Survival rate of Penaeus vannamei against V. parahaemolyticus in relation to salinity and bacterial concentration (CFU/ml).*

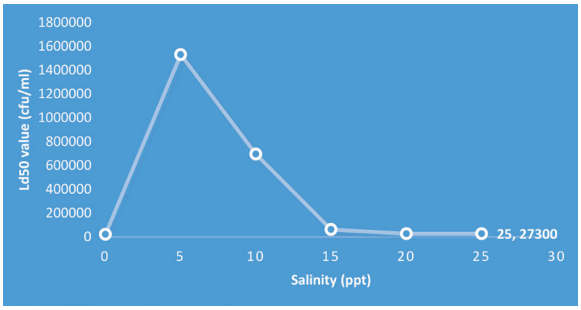

Figure 5. *Interaction of Vibrio parahaemolyticus pathogenicity in relation to salinity and LD_{50} value.*

Table 2. *Antimicrobial activity of crude stem juice and ethanol extract of Tinospora cordifolia.*

Test organism	Material	Inhibition zone (mm)	
Vibrio parahaemolyticus	Ethanol extract (mg residue/ml solvent)	Mueller-Hinton agar	TCBS agar
	50	7±0.12	7±0.32
	100	8±0.32	8±0.03
	150	8±0.21	13±0.53
	200	14±0.43	13±0.92
	250	14±0.23	15±0.43
	-ve control	0±0.00	0±0.00
	+ve control	9±0.02	9±0.02
	Crude stem juice (%)		
	10	6.5±0.02	7±0.20
	25	7±0.27	7±0.50
	50	7±0.56	7±0.25
	75	10±0.23	9±0.75
	100	10±0.34	10±0.25
	-ve control	0±0.00	0±0.00

density, no mortality was observed *(25)*. In *P. vannamei* mortality in immersion challenges of isolate began in treatments with 1×10^4 CFU mL^{-1} concentration *(36,26)*.

The study conducted with respect to the interaction of bacterial pathogenicity with different salinity 0, 5, 10, 15, 20, 25 ppt the survival rate was significantly higher in shrimp juvenile in the salinity of 5 and 10 ppt (Figure 4) and also maximum resistance against bacterial infection in *P. vannamei* showed that 5 ppt to 10 ppt with higher CFU/shrimp value (Figure 5). Whereas in the case of 0 ppt and as the salinity increases like in the case of 15, 20, and 25 ppt salinity CFU/shrimp value decreases, and bacterial pathogenicity increases. 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 due to the fact that in 0 ppt *P. vannamei* are not getting their minimum requirement for optimum growth as it can grow successfully at salinities of 5 to 35 ppt *(38)*. Further study may be conducted in this regard for the increased pathogenicity of the bacteria at 0 ppt.

The zones of inhibition in diameter (mm) recorded for ethanol and, crude stem juice is depicted in Table 2. The highest zone of inhibition was observed against *Vibrio parahaemolyticus* with *Tinospora cordifolia* stem ethanol extract 15±0.43 mm of the clear zone at 250 mg mL^{-1} concentration in TCBS agar followed by 14±0.23 mm of the clear zone at 250 mg mL^{-1} concentration in Mueller-Hinton agar were observed. Whereas, in the case of crude stem juice 10±0.34 mm of the clear zone at 100% concentration in Mueller-Hinton agar and 10±0.25 mm of the clear zone at 100% concentration in TCBS agar were observed. The antibacterial sensitivity test revealed that both the ethanol extracts and the crude stem juice exhibited antibacterial activity against *V. parahaemolyticus* but from the results, it dictates that the greater activity resides in ethanol stem extracts of plant compared to 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 marcescens* tested bacteria *(39)*. It can be interpreted that the antibacterial activity against microorganisms is due to any one or more alkaloids of the plants *(24)*.

CONCLUSIONS

In conclusion, we found that the reason for the mass mortality of juvenile shrimp *P. vannamei* in Kakinada, Andhra Pradesh, India, was *Vibrio parahaemolyticus* strain KKD 02. It caused 100% mortality during bath challenge in 10^7 CFU mL^{-1} and 10^6 CFU mL^{-1} concentrations with-

in 24 h and, was positive for two virulence genes *tdh* and *trh*. The study conducted with respect to the interaction of bacterial pathogenicity with different salinity 0, 5, 10, 15, 20, 25 ppt showed that salinity range from 5 ppt to 10 ppt was connected to the maximum resistance against bacterial infection in *P. vannamei* with higher CFU/shrimp value. Whereas in the case of 0 ppt and as the salinity increases like in the case of 15, 20 and 25 ppt salinity CFU/shrimp value decreases and bacterial pathogenicity increases. It is revealed that both 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 to examine 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.

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