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Isolation and Characterization of Vibrio parahaemolyticus in the hepatopancreas of cultured white pacific shrimp - Litopenaeus vannamei

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ABSTRACT

Vibrio parahaemolyticus is the most common species among crustaceans, often causing various diseases and significant losses in aquaculture. Acute hepatopancreatic necrosis disease (AHPND) is a newly emerging shrimp disease that has severely damaged the global shrimp industry. This species of bacteria is associated with gastrointestinal illness in humans and has been implicated in foodborne disease. The present study carried out, isolation and characterization of pathogenic bacterial flora isolated from the infected hepatopancreas of vannamei, obtained from various aquafarms in Andhra Pradesh, India, on 11^{th} June 2018. The collected samples were plated on TCBS- (Thiosulfate-Citrate-Bile salt-Sucrose) agar medium and Hi -Chrome vibrio, as described in Bergey's manual of systematic bacteriology. Isolated colonies were subjected to the following tests- microscopic examination, growth at different temperatures, growth at different NaCl concentrations, and biochemical tests. Further purity, maintenance, and propagation of purified cultures were done. The microbial culture was identified using 16s rRNA molecular technique. Phylogenetic Evolutionary analyses and distance matrix were conducted in MEGA7.In the present study, different samples were screened, a total of three green colonies (V44, V45, V46) were isolated, identified by biochemical tests and genetic identification as Vibrio parahaemolyticus. A systematic methodology has been developed to isolate and characterize Vibrio sp. from diseased shrimp and identify them by genetic analysis.

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INTRODUCTION

According to the Food and Agriculture Organization (FAO)," Aquaculture means farming of fish, crustaceans, molluscs, aquatic plants. Aquaculture involves cultivating freshwater and saltwater populations under controlled conditions. Particular techniques include aquaponics and integrated multi-trophic aquaculture, both of which integrate fish farming and aquatic plant farming (FAO, 2002). Fishing is a major industry in India, with the country exporting 13,77,244 metric tonnes of seafood worth US$7.08 billion (45,106.89 crores) in 2017-18, with shrimp being the top item for export worth US$4.84

billion. (30,868.17 crore). The state of Andhra Pradesh accounts for 42% of total shrimp farming in India, with 64,283 hectares (MPEDA, 2018). P. vannamei and P. monodon are the primary species of farm-raised shrimp cultivated in both the Eastern and the Western hemispheres. (Holthuis, 1980) Cultivation of shrimp is faced with problems of water quality, unsatisfactory practices, and shrimp diseases caused by bacteria and viruses, resulting in great economic losses (Lightner and Redman, 1998). Pathogenic bacteria of Vibrio sp. such as V. harveyi, V. anguillarum, V. parahaemolyticus, and V. vulnificus have been involved in mortality of cultured shrimp in hatcheries (Mohney *et al.*, 1994; Baticados *et al.*, 1990).In shrimp cultures, Vibrio parahaemolyticus is the major pathogen isolated from the infected hepatopancreas of shrimp (Shanmugasundaram *et al.*, 2015). Hence the present investigation is done on isolation and identification of Vibrio parahaemolyticus from various sources of shrimp aquaculture farms.

MATERIALS AND METHODS

Collection of samples

In the present study, infected shrimp were collected on 11^{th} June 2018 from shrimp aquaculture farms (Kallanka, Vendra, and Juvvalapalem), which are located in East and West Godavari Dist. of Andhra Pradesh, India ,Tables 1, 2 and 3.

Isolation of colonies from infected shrimp

The hepatopancreas of infected shrimp was dissected and isolated for pathogenic bacteria. The samples were serially diluted 10^{-1} to 10^{-9} factor, and 10^{-5} to 10^{-7} dilutions are spread over sterilized TCBS Petri plates (Thiosulphate Citrate Bile salt Sucrose). The Petri dish is placed at 37-45^{o}C overnight in an incubator. The incubation period was maintained at 37^{o}C for 24-48 hours. For vibrio species identification, the colonies isolated were then purified and stored in TCBS agar slants for further studies.

Identification of selected Vibrio isolates

Identification and further characterization of vibrio sp.(V44, V45, V46) grown on TCBS agar and Hi -Chrome vibrio agar was done by the procedure described in Bergey's manual of systematic bacteriology with following tests carried out: microscopic examination, growth at different temperatures, growth at different NaCl concentration and biochemical tests (Alsina and Blanch, 1994; Hidaka, 1968; Macian *et al.*, 1996; Elliot *et al.*, 2001; Baumann *et al.*, 1972; Ottaviani *et al.*, 2003).

Microscopic examination

Purity, morphology, and microscopic examination of Vibrio isolates was confirmed by performing gram staining for which a single colony of each isolate was stained as per standard protocol and viewed under oil immersion for a similar type of cells.

Physiological Characteri zation of Isolates

Once the purity of culture was confirmed, each isolates were further assessed for growth at different temperatures and salt concentration.

Growth of isolates at (10^{o}C, 30^{o}C, 40^{o}C)

The isolates were examined for growth in nutrient agar plates at 10^{o}C for 7 days and 30^{o}C,40^{o}C for 24-48h. 1 % of vibrio isolates culture were mixed with 20ml nutrient agar, and growth was observed at different temperatures was noted as positive or negative.

Effect of NaCl concentration on isolates growth

The isolates were inoculated with different NaCl concentrations (0%,3%,6%, 7% and 8%) in nutrient agar plates and incubated at 37^{o}C for 24-48h. Culture plates were observed for the presence or absence of growth.

Biochemical characterization of isolates

Oxidase test

The test is used to identify bacteria that produce cytochrome C oxidase, an enzyme of bacterial electron transport chain. The culture was placed on slide using inoculation needle, and drop of N, N, N′, N′-tetramethyl-p-phenylenediamine solution was added on to the culture and observed for the oxidized purple colour end product which indicates a positive result.

Catalase test

The test was performed on isolated cultures to determine the degradation of hydrogen peroxide by producing enzyme catalase. The culture was placed on slide using inoculation needle, and a drop of 3% hydrogen peroxide solution was added on to the culture and observed for the evolution of bubbles, which indicates a positive result.

KOH String Test

It relies on the differential resistance to 3% potassium hydroxide between gram-positive and negative cells, where a portion of a colony is mixed with a small volume of 3% KOH. If the cells lyse, the liberated cellular DNA makes the mixture viscous or "stringy."

Arginine hydrolysis

Arginine hydrolysis broth was inoculated with isolated cultures and incubated at 37^{o}C for 48h. After

incubation, 3-5 drops of Nessler's reagent were added to each tube and observed for change in colour (yellow to orange), indicating a positive result.

Lysine hydrolysis

It is used to detect specific enzyme production. Medium is inoculated with bacteria which ferments dextrose, causing a lowering of pH in the medium due to acid production and color of the indicator changes from purple to yellow. Acidic condition stimulates decarboxylase activity.

Ornithine hydrolysis

It is done to detect the production of ornithine decarboxylase. Glucose the fermentable carbohydrate, which during the early stages of incubation, is fermented by microbe with acid production, resulting in a colour change to yellow due to change in pH.

Nitrate reduction test

Isolates were added in trypticase nitrate broth and incubated at 37^{o}C for 24h. After incubation, 0.5 mL of sulphanilic acid and α-naphthylamine were added into the tubes. The appearance of red or pink colour indicates a positive result for nitrate reduction.

Indole test

Isolates were inoculated in tryptophan broth, incubated at 37^{o}C for 24h. After incubation, 0.5 ml of Kovac's reagent was added. Pink colour ring in the top layer indicates a positive result.

ONPG Test

The microbe is selected from a medium with high lactose concentration and is then inoculated into the ONPG Broth. If the microbe contains beta-galactosidase, the enzyme will then split the beta-galactoside bond, releasing o-nitrophenol, which is a yellow-colored compound.

Citrate utilization test

The isolates were inoculated in Simmons citrate agar, incubated at 37^{o}C for 24h. After incubation, the appearance of blue color indicates a positive result for citrate utilization.

Hydrogen Sulfide (H2S) Production Test

In this test, sulfur-containing compounds reduced to sulfides during metabolism is tested. The presence of sulphide, on reaction with iron compounds, produces FeS a black precipitate.

Urease test

Microbe producing an exoenzyme urease in a differential medium of Urease broth that hydrolyzes urea to ammonia and carbon dioxide is tested. Urea broth is designed to test urease positive microbes.

VP (Voges Proskauer) test

Isolates were added to MRVP broth and incubated for 37^{o}c for 48 h. For VP test Barrit's reagent was added to the tube. The pink colour indicates VP positive**.**

Gelatin liquefaction

In this test, microbes producing gelatinase that liquefy gelatin are detected. Gelatin hydrolysis indicates the presence of gelatinases.

Carbohydrates fermentation by isolates

Sugars were used to determine the fermentation profile and further characterization of *Vibrio isolates*. Carbohydrates basal media was prepared, and pH adjusted to 7- 7.4. Phenol red was added as an indicator. Sugars were mixed to the basal media (500mg of sugar in 50mL of basal media) and autoclaved. Each tube was inoculated with 50μL of inoculum in 250μL of sugar basal media. Control using sterile water was prepared to compare colour change.

Maintenance and propagation of cultures

Isolates of *Vibrio sp.*were maintained at -70^{o}C in glycerol stocks in triplicate for use in an experiment at different stages.

Purity of cultures

Vibrio species isolates were regularly tested for microscopic examination and catalase tests for confirmation and presence of contamination, if any.

Identification of microbial culture using 16s rRNA based molecular technique method

Genomic DNA was isolated, and quantity was measured using a nanodrop spectrophotometer, and the quality was determined using 2% agarose gel**.** A single band of high-molecular-weight DNA has been observed. 16S rRNA gene was amplified by 16SrRNAF and 16SrRNAR primers. A single discrete PCR amplicon band of 1500 bp was observed when resolved on an agarose gel. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with forwarding primer and reverse primers using BDT v3.1. Cycle sequencing kit on ABI 3730xl genetic analyzer. A consensus sequence of the 16S rRNA gene was generated from forward and reverse sequences. 16S rRNA gene sequence was used to carry out BLAST with the database of the NCBI Genbank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program

Table 1: Sample no -V44

Date	Name	Place	Pond size	DOC	Length
06-09-2018	Anil Kumar	Kallanka	4AC	45 Days	14cm
Weight	HP Weight	WSSV	Harvest days	Severity	
19 gm	650 mg	negative(physical)	still culture	+	

Table 2: Sample no-V45

Date	Name	Place	Pond size	DOC	Length
11/6/2018	SR Raju	Vendra	4AC	43 Days	9cm
Weight	HP Weight	WSSV	Harvest days	Severity	
10.40gm	500mg	ehp negative (RT-PCR)	still culture	++	

Table 3: Sample no-V46

Date	Name	Place	Pond size	DOC	Length
13/6/2018	V Bhaskar Raju	Juvvalapalem	7AC	46 Days	10.5cm
Weight	HP Weight	WSSV	Harvest days	Severity	
7.60gm	350mg	positive (physical)	still culture	++	

Doc- date of collection, HP-hepatopancreas, WSSV-white spotsyndrome virus

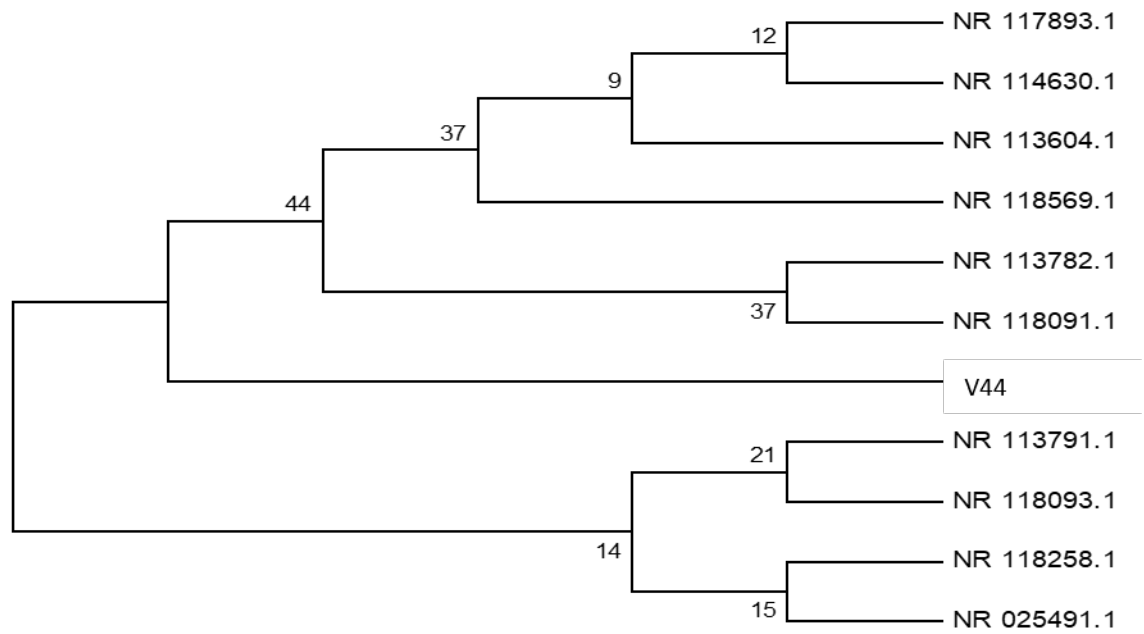

Figure 1: Phylogenetic Tree

Clustal W. Distance matrix was generated, and the phylogenetic tree was constructed by using MEGA7.

Phylogenetic tree

The evolutionary history was inferred using the Neighbor-Joining method (Nei and Saitou, 1987) The optimal tree with the sum of branch length = 0.09337190 was shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site. The analysis involved 6 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions con-

Table 4: Morphological, physiological and biochemicalcharacterization of isolates

Characteristics	V44	V45	V46
Morphological characterization			
Colour	Green	Green	Green
Shape	Rod	Rod	Rod
Colony size (μm)	3	3	3
Colony Form	Circular	Circular	Circular
Motility	Motile	Motile	Motile
Grams reaction	Negative	Negative	Negative
Physiological characterization			
Growth at different temperature			
10^{o}C	-	-	-
30^{o}C	+	+	+
40^{o}C	+	+	+
Growth at different NaCl concentration			
0%	-	-	-
3%	+	+	+
6%	+	+	+
7%	+	+	+
8%	+	+	+
Biochemical characterization			
Growth on TCBS	Green	Green	Green
Growth on Hichrome vibrio agar	Green	Green	Green
Cytochrome oxidase	+	+	+
Catalase	+	+	+
KOH string	+	+	+
Arginine dihydrolase	-	-	-
Lysine decarboxylase	+	+	+
Ornithine decarboxylase	+	+	+
Nitrate reduction	+	+	+
Indole	+	+	+
β- galactosidase (ONPG)	-	-	-
Citrate utilization	-	+	+
Production of H2S	-	-	-
Urease	+	+	+
VP test	-	-	-
Gelatin liquification	+	+	+
Acid from			
Glucose	+	+	+
Mannitol	+	+	+
Arabinose	+	+	+
Sucrose	-	-	-
Sorbital	-	-	-
Cellobiose	-	-	-
Inositol	-	-	-
Mannose	+	+	+

$V, variable; ONPG, ortho-nitrophenyl-\beta-galactoside; VP, Voges-Proskauertest; +, positive; -, negative$

Table 5: Estimates of Evolutionary Divergence between Sequences

	Distance matrix	1	2	3	4	5	6	7	8	9	10	11	
1	V44		58.869	66.398	49.177	58.869	49.537	66.398	49.177	58.869	58.869	49.537	1
2	NR_118569.1	0.000		0.002	0.001	0.000	0.001	0.002	0.001	0.000	0.000	0.001	2
3	NR_113791.1	0.000	0.002		0.003	0.002	0.002	0.000	0.003	0.002	0.002	0.002	3
4	NR_113782.1	0.000	0.001	0.004		0.001	0.002	0.003	0.000	0.001	0.001	0.002	4
5	NR_113604.1	0.000	0.000	0.002	0.001		0.001	0.002	0.001	0.000	0.000	0.001	5
6	NR_118258.1	0.000	0.001	0.001	0.002	0.001		0.002	0.002	0.001	0.001	0.000	6
7	NR_118093.1	0.000	0.002	0.000	0.004	0.002	0.001		0.003	0.002	0.002	0.002	7
8	NR_118091.1	0.000	0.001	0.004	0.000	0.001	0.002	0.004		0.001	0.001	0.002	8
9	NR_117893.1	0.000	0.000	0.002	0.001	0.000	0.001	0.002	0.001		0.000	0.001	9
10	NR_114630.1	0.000	0.000	0.002	0.001	0.000	0.001	0.002	0.001	0.000		0.001	10
11	NR_025491.1	0.000	0.001	0.001	0.002	0.001	0.000	0.001	0.002	0.001	0.001		11
		1	2	3	4	5	6	7	8	9	10	11	

Table 6: Estimates of Evolutionary Divergence between Sequences

	Distance matrix	1	2	3	4	5	6	7	8	9	10	11	
1	V45		58.869	66.398	49.177	58.869	49.537	66.398	49.177	58.869	58.869	49.537	1
2	NR_118569.1	0.000		0.002	0.001	0.000	0.001	0.002	0.001	0.000	0.000	0.001	2
3	NR_113791.1	0.000	0.002		0.003	0.002	0.002	0.000	0.003	0.002	0.002	0.002	3
4	NR_113782.1	0.000	0.001	0.004		0.001	0.002	0.003	0.000	0.001	0.001	0.002	4
5	NR_113604.1	0.000	0.000	0.002	0.001		0.001	0.002	0.001	0.000	0.000	0.001	5
6	NR_118258.1	0.000	0.001	0.001	0.002	0.001		0.002	0.002	0.001	0.001	0.000	6
7	NR_118093.1	0.000	0.002	0.000	0.004	0.002	0.001		0.003	0.002	0.002	0.002	7
8	NR_118091.1	0.000	0.001	0.004	0.000	0.001	0.002	0.004		0.001	0.001	0.002	8
9	NR_117893.1	0.000	0.000	0.002	0.001	0.000	0.001	0.002	0.001		0.000	0.001	9
10	NR_114630.1	0.000	0.000	0.002	0.001	0.000	0.001	0.002	0.001	0.000		0.001	10
11	NR_025491.1	0.000	0.001	0.001	0.002	0.001	0.000	0.001	0.002	0.001	0.001		11
		1	2	3	4	5	6	7	8	9	10	11	

Table 7: Estimates of Evolutionary Divergence between Sequences

	Distance matrix	1	2	3	4	5	6	7	8	9	10	11	
1	V46		58.869	66.398	49.177	58.869	49.537	66.398	49.177	58.869	58.869	49.537	1
2	NR_118569.1	0.000		0.002	0.001	0.000	0.001	0.002	0.001	0.000	0.000	0.001	2
3	NR_113791.1	0.000	0.002		0.003	0.002	0.002	0.000	0.003	0.002	0.002	0.002	3
4	NR_113782.1	0.000	0.001	0.004		0.001	0.002	0.003	0.000	0.001	0.001	0.002	4
5	NR_113604.1	0.000	0.000	0.002	0.001		0.001	0.002	0.001	0.000	0.000	0.001	5
6	NR_118258.1	0.000	0.001	0.001	0.002	0.001		0.002	0.002	0.001	0.001	0.000	6
7	NR_118093.1	0.000	0.002	0.000	0.004	0.002	0.001		0.003	0.002	0.002	0.002	7
8	NR_118091.1	0.000	0.001	0.004	0.000	0.001	0.002	0.004		0.001	0.001	0.002	8
9	NR_117893.1	0.000	0.000	0.002	0.001	0.000	0.001	0.002	0.001		0.000	0.001	9
10	NR_114630.1	0.000	0.000	0.002	0.001	0.000	0.001	0.002	0.001	0.000		0.001	10
11	NR_025491.1	0.000	0.001	0.001	0.002	0.001	0.000	0.001	0.002	0.001	0.001		11
		1	2	3	4	5	6	7	8	9	10	11	

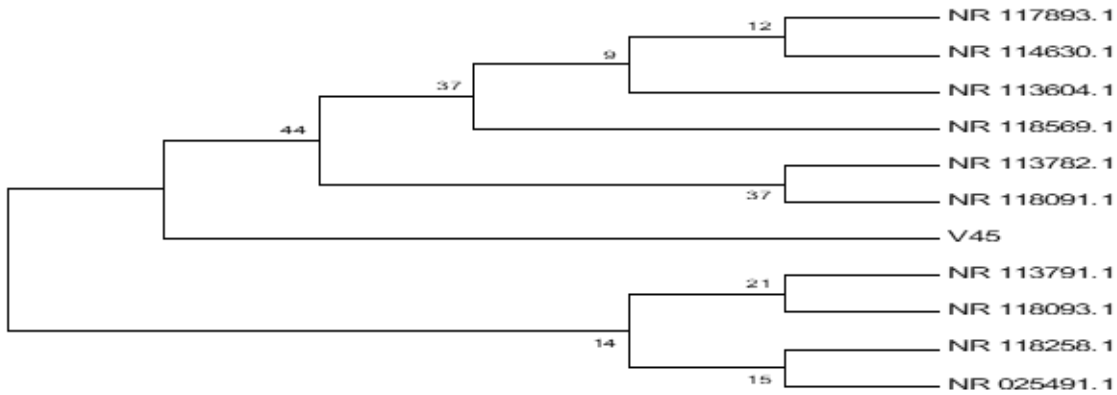

Figure 2: PhylogeneticTree

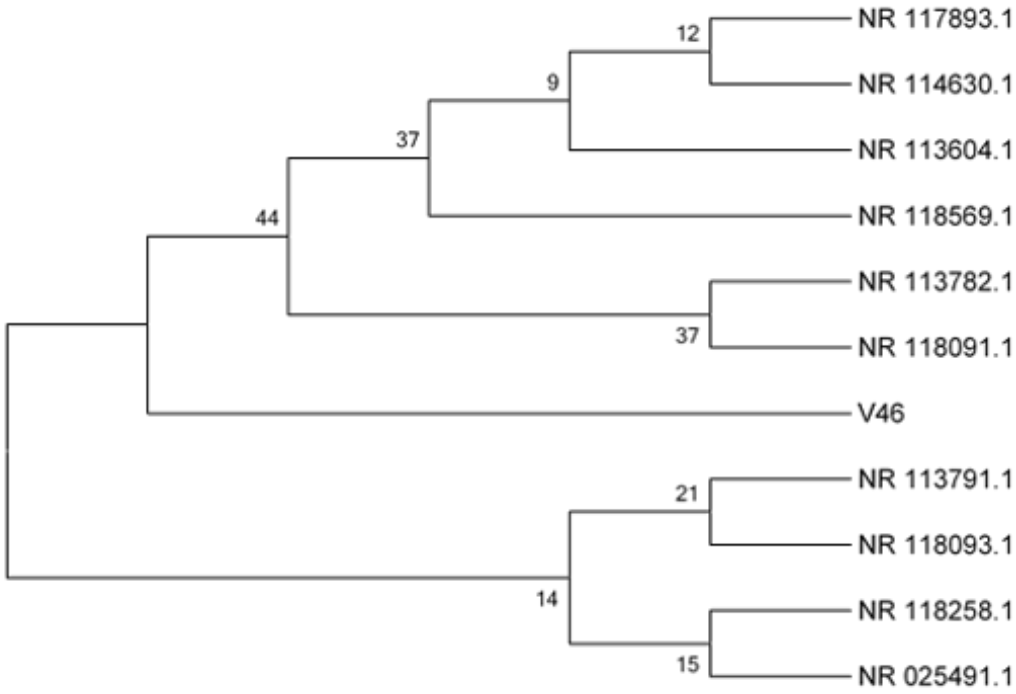

Figure 3: Phylogenetic Tree

taining gaps and missing data were eliminated. There were a total of 5851 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al.*, 2016).

Distance matrix

The number of base substitutions per site from between sequences is shown. Analyses were conducted using the Maximum Composite Likelihood model (Nei and Saitou, 1987). The analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 5851 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Felsenstein, 1985).

RESULTS AND DISCUSSION

Biochemical and genetic identification

Table 4 describes the Morphological, physiological, and biochemical characterization of isolates. The identification of microbial culture using 16s rRNA based molecular technique was carried out, and the results are mentioned below(Figures 1, 2 and 3 and Tables 5, 6 and 7). Based on sequence homology and phylogenetic analysis, the microbial culture labeled V44, V45 &V46 were found to be Vibrio parahaemolyticus**.**

Shrimp was harvested from different ponds of varying sizes. Its length, weight, and hepatopancreas weight were checked. Harvest was done from still culture, and severity of disease was assessed.the

hepatopancreas were dissected, suitable dilutions made, and plating was done on TCBS agar for isolation of vibrio sp. Morphology, physiological, and biochemical characterization of isolates was done to identify specific tests, which is typical for Vibrio sp.

Growth was observed at a mesophilic temperature of 30^0C, 40^0C, but not at 10^0Cand microbes grew at a salt concentration of 3-8 % but not 0% for all the isolates. Positive results on TCBS, HiChrome vibrio agar, cytochrome oxidase, catalase, KoH, lysine, ornithine, nitrate, indole urease, gelatin liquefaction, and few sugars was observed. Negative results were observed for arginine, ONPG, Citrate, VP, and few sugars tests. Identification of isolates was made using 16s rRNA based Molecular technique. The results of the technique identified bacteria from harvested shrimp as Vibrio parahaemolyticus. The research attempt is to identify bacteria, causing the disease for the shrimp and isolate and identify them for specific treatment.

CONCLUSIONS

In summary, the shrimp harvested instill cultures were studied for pathogenic bacteria of Vibrio sp.The vibrios were extensively characterized and confirmed as vibrio paraheamolyticus by genetic evaluation. This species is a source of water contamination and can cause gastroenteritis if taken as part of a food chain in humans. However, further study is required to evaluate them in animal experiments and in-vitro cell cultures.

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