

Distribution of *Vibrio parahaemolyticus* in Farmed Shrimp *Penaeus vannamei*, Farm Water and Sediment

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Abstract

The halophilic marine bacterium *Vibrio parahaemolyticus* is a zoonotic pathogen associated with wild-caught and farmed shrimp. The bacterium is an important cause of gastroenteritis associated with the consumption of raw or undercooked seafood. In the present study, the prevalence and human pathogenic potential of *Vibrio parahaemolyticus* in *Penaeus vannamei* (tissue and hepatopancreas) and the farm environment (water and sediment) was investigated by conventional culture and molecular techniques. The total *Vibrio* counts of *P. vannamei* ranged from <1 CFU/mL in hemolymph to 7.61 log CFU/g in the hepatopancreas. The sediment samples consistently showed the counts of 6-7 log CFU/g, while the pond water had *Vibrio* counts in the range of 2-3 log CFU/ml. Of 120 *Vibrio* isolates identified, 87 were confirmed as *V. parahaemolyticus* based on the *toxR* and *tlh* gene-specific PCR. The virulence marker gene *tdh* was not detected in any of the isolates, while the *trh* gene was detected in 3 (3.6%) isolates. Although the incidence of pathogenic *V. parahaemolyticus* in farmed *P. vannamei* is low, the high numbers of total *vibriosis* and *V. parahaemolyticus* demand constant monitoring of animals and the farm environment for human pathogenic strains of *V. parahaemolyticus*.

Keywords: *Vibrio parahaemolyticus*, pathogenic, zoonotic, shrimp, virulence

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INTRODUCTION

Among various pathogenic vibrios, *Vibrio parahaemolyticus* is frequently associated with seafood-borne human gastroenteritis¹. Consumption of raw or undercooked shellfish is generally responsible for human infections by *V. parahaemolyticus*. However, all strains of *V. parahaemolyticus* are not pathogenic. The strains that produce a thermostable direct hemolysin (TDH) or a TDH-related hemolysin (TRH) are considered pathogenic to humans². The TDH-producing *V. parahaemolyticus*, which comprises less than 1% of the strains of *V. parahaemolyticus*, is more frequently involved in human infections. Isolates producing TRH alone were first reported to be involved in the outbreak of human gastroenteritis from a patient in the Republic of Maldives³. Studies have shown that TRH-positive isolates are more abundant, sometimes as high as 15% of the total *V. parahaemolyticus*, and usually cause gastrointestinal infections which are less severe in nature^{4,5}. Nevertheless, both TDH- and TRH-positive *V. parahaemolyticus* are considered human pathogens⁵. Infections with *V. parahaemolyticus* usually occur during warm summer months in temperate countries. However, in tropical coastal waters with a more or less stable temperature throughout the year, *V. parahaemolyticus* numbers in seafood are temperature independent but are influenced by fluctuations in salinity⁶. Infections generally occur when seafood containing 10^7 - 10^8 CFU/ml *V. parahaemolyticus* is consumed⁷. Several studies have reported the occurrence of TDH- and TRH-positive *V. parahaemolyticus* from seafood in India^{6,8-12}. Apart from gastroenteritis, *V. parahaemolyticus* can cause wound infections¹³. *V. parahaemolyticus* is also a pathogen of fish and shellfish, and has been associated with disease in milkfish (*Chanos chanos*) in the Philippines¹⁴, farmed sea bass (*Dicentrarchus labrax*) in Tunisia¹⁵, red disease in *Penaeus monodon* in India¹⁶. The significance of *V. parahaemolyticus* as a serious pathogen of farmed shrimp was realized with the establishment of its association with the acute hepatopancreatic necrosis disease AHPND in shrimp^{17,18}. With the association of *V. parahaemolyticus* with diseases in farmed fish and shrimp, the bacterium has been recognized as a zoonotic human pathogen. Considering the

human health threat this bacterium poses, it is important to routinely monitor the prevalence of *V. parahaemolyticus* in farmed shrimp as part of risk assessment studies. In this context, the study was designed to understand the prevalence of total and human pathogenic *V. parahaemolyticus* in shrimp farm environments on the West Coast in India.

MATERIALS AND METHODS

Sample collection and preparation

Twenty-one samples comprising of shrimp (9), water (6), and sediment (6) analyzed in this study were collected from three shrimp (*P. vannamei*) aquaculture farms in Saphale (19.5708° N, 72.8309° E) and Dahanu (19.9903° N, 72.7397° E) in Palghar district of Maharashtra, India. Fresh shrimp samples were collected directly from the ponds in sampling bags and immediately transported to the laboratory in chilled condition. The water and sediment samples of the ponds were collected from three different locations within a pond and pooled. The total *Vibrio* counts of shrimp hemolymph, tissue, hepatopancreas, as well as water and sediment samples of the respective ponds were analyzed following standard methods¹⁹, with some modifications. The samples were surface plated directly on selective agar plates for the quantification of presumptive *V. parahaemolyticus* without the selective enrichment. The salinity and water temperature were measured before the collection of samples.

Shrimp samples

Different parts of the shrimp i.e., hepatopancreas, tissue, and hemolymph were processed for the isolation and quantification of *V. parahaemolyticus*. The hemolymph was collected from three live animals from a single pond using sterile syringes and pooled. From this, 0.1 mL was directly surface plated on thiosulphate citrate bile salt sucrose (TCBS) agar. Tissue and hepatopancreas were sampled from three different shrimps from the same pond and pooled and one gram of the pooled sample was processed further. Briefly, one gram of the hepatopancreas or the tissue was homogenized in 9 mL of sterile saline solution (2% NaCl w/v), serially 10-fold diluted in saline, and 0.1 mL of the dilutions was spread plated on TCBS agar plates in duplicate. Water and sediment samples

In the case of water samples, 0.1 mL was spread plated directly on TCBS agar plates. The sediment sample (10 g) was mixed with 90 ml of 2% (w/v) saline, vortexed for 2 min, and allowed to settle for 5 min. The supernatant was 10-fold serially diluted in 2% saline and 0.1 mL from each dilution was spread plated on TCBS agar plates²⁰.

The inoculated plates were incubated at 35°C for 18-24 h. Green colonies typical of *V. parahaemolyticus*, 3-5 from each TCBS plate, were picked and streaked on Luria Bertani agar plates containing 2% NaCl and incubated for 24 h at 35°C. The bacterial isolates were subjected to presumptive identification of *Vibrio* spp. using biochemical reactions such as the production of cytochrome oxidase, catalase, resistance to 10 µg concentration of the vibriostatic agent O/129 (2,4-diamino-6,7-diisopropyl pteridine), growth at different concentrations of salt (0, 3, 6, 8 and 11% w/v NaCl) and glucose fermentation tests.

Molecular characterization

Vibrio isolates from the samples were subjected to *V. parahaemolyticus* species-specific PCR targeting the *toxR* and *tlh* genes. To detect pathogenic strains of *V. parahaemolyticus*, the isolates were subjected to PCR specific for the thermostable direct hemolysin gene (*tdh*) and the TDH-related hemolysin gene (*trh*) (Table 1). For preparation of DNA for PCR, a single bacterial colony was suspended in 200 µl 1×TE (10 mM Tris pH 8.0; 1mM EDTA) buffer in a 1.5 mL microcentrifuge tube and vortexed. The bacterial suspension was lysed by placing the tube in a dry bath at 98°C for 10 min, followed by placing it on ice for 5 minutes. The lysate was centrifuged at 10,000 rpm for 2 min and the resultant

supernatant was used as the DNA template in PCR.

PCR was performed in 30 µl reaction volumes consisting of 3 µl of 10X buffer (DSS Takara Bio India Pvt. Ltd.), 200 µM concentrations of each of the four dNTPs, 10 pico moles of each of forward and reverse primers, and 1 U of *Taq* DNA polymerase (DSS Takara Bio India Pvt. Ltd.) and 3 µl of template DNA. The amplifications were performed in SimpliAmp™ thermal cycler (Thermo Fisher Scientific, USA). *V. parahaemolyticus* AQ 4037 (*trh*⁺) and SY O3:K6 (*tdh*⁺) were used as the positive controls, while sterile distilled water was used as the negative control. The thermocycling conditions for the amplification of target genes (*toxR*, *tlh*, *trh* and *tdh*) were essentially the same as previously described^{21,22}. The PCR amplicons obtained were resolved on 1.5% agarose gels by electrophoresis. The ethidium bromide-stained gels were then visualized and photographed using a gel documentation system (UVP, CA, USA).

RESULTS AND DISCUSSION

Isolation and characterization of *V. parahaemolyticus*

The presence of pathogenic and non-pathogenic strains of *V. parahaemolyticus* has been reported from wild-caught seafood in India²³⁻²⁵. Although *V. parahaemolyticus* has been considered as an important human pathogen, the bacterium is also an important shrimp pathogen. Studies have reported *V. parahaemolyticus* infections of *P. monodon* and *P. vannamei* at various life stages, from post-larvae to adult shrimps^{26,27}. The zoonotic potential of *V. parahaemolyticus* makes it an important pathogen of public health significance as well. *V. parahaemolyticus* in shrimp

Table 1. Oligonucleotide primers used in the study

Target gene	Primer sequence (5'-3')	Amplicon size (bp)	Reference
<i>toxR</i>	F: GTC TTC TGA CGC AAT CGT TG	368	21
	R: ATA CGA GTG GTT GCT GTC ATG		
<i>tdh</i>	F: AAA GCG GAT TAT GCA GAA GCA CTG	269	
	R: GCT ACT TTC TAG CAT TTT CTC TGC		
<i>trh</i>	F: GTA AAG GTC TCT GAC TTT TGG AC	500	22
	R: TGG AAT AGA ACC TTC ATC TTC ACC		
<i>tlh</i>	F: TTG GCT TCG ATA TTT TCA GTA TCT	450	
	R: CAT AAC AAA CAT ATG CCC ATT TCC G		

farm environment can lead to persistent infections and crop losses^{28,29}, while its presence in processed shrimp can lead to consignment rejections³⁰. In this context, it is important to monitor farmed shrimp for the presence of *V. parahaemolyticus* in general and human pathogenic (*tdh*- and/*trh*-positive) *V. parahaemolyticus* in particular.

Twenty-one samples collected from three different shrimp farms were analyzed for the presence of *V. parahaemolyticus*. A total of 120 isolates, identified as *Vibrio* spp. by biochemical tests, were recovered from the samples. Of these, 31 isolates were from shrimp tissue, 59 were from shrimp hepatopancreas, and 15 each were from

water and sediment samples (Table 2). These isolates were subjected to *V. parahaemolyticus*-specific PCR targeting *toxR* and *tlh* genes. Of 120 isolates, 87 (72.5%) isolates were confirmed as *V. parahaemolyticus* by PCR (Fig. 1A & 1B). The difference in the nucleotide sequences of *toxR* gene among different *Vibrio* spp. has been utilized to develop species-specific primers²¹. The *toxR* specific PCR has been used by several investigators to identify *V. parahaemolyticus* isolated from seafood^{6,31,32}. The prevalence studies of *V. parahaemolyticus* from India reported 89% in seafood in general³³ and 80% in shrimps³⁴.

Studies on farmed shrimp and the

Table 2. Details of the total (and pathogenic *Vibrio parahaemolyticus* isolated from shrimp, water and sediment samples

Sample Type	No. of <i>Vibrio</i> [‡] isolates recovered	No. <i>toxR</i> positive	No. <i>tlh</i> positive	No. <i>trh</i> positive	No. <i>tdh</i> positive
Hepatopancreas	59	50	50	2	0
Tissue	21	21	21	0	0
Water	15	7	7	0	0
Sediment	15	9	9	1	0
Total	120	87	87	3	0

[‡]Based on the biochemical tests

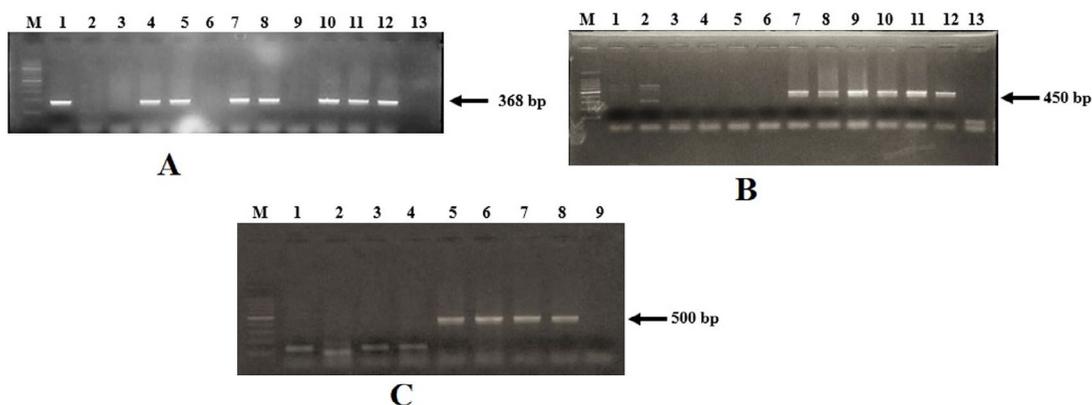


Fig 1. Agarose gel electrophoresis for the vibrio isolates

Detection of *toxR* gene (A). Lane M: 1 kb DNA ladder (Thermo Fisher Scientific, USA); lanes 1-11: presumptive *V. parahaemolyticus* isolates from shrimp, water and sediment samples; lane 12: positive control (*V. parahaemolyticus* AQ 4037); lane 13: negative control

Detection of *tlh* gene (B). Lane M: 100 bp DNA ladder (Thermo Fisher Scientific, USA); lanes 1-3: isolates from sediment; lanes 4-5: isolates from water; lanes 6-8: isolates from hepatopancreas; lanes 9-11: isolates from shrimp tissue; lane 12: positive control (*V. parahaemolyticus* AQ 4037); lane 13: negative control.

Detection of *trh* gene (C). Lane M: 100 bp DNA ladder (Thermo Fisher Scientific, USA); lanes 1-2: isolates from shrimp tissue; lanes 3-4: isolates from water; lanes 5-6: isolates from hepatopancreas; lanes 7: isolates from sediment; lane 8: positive control (*V. parahaemolyticus* AQ 4037); lane 9: negative control.

farm environments have reported that the *V. parahaemolyticus* is commonly associated with shrimps, although their numbers can vary depending on the temperature, salinity, and other abiotic and biotic factors^{35,36}. *V. parahaemolyticus* was reported to be the dominant *Vibrio* spp. after *V. harveyi* and *V. splendidus* in the hepatopancreas of pond-reared *Penaeus monodon* juveniles affected with luminous vibriosis³⁷. Other studies have reported *V. parahaemolyticus* as the predominant *Vibrio* spp. in farmed *Penaeus monodon*^{38,39}. Sanathkumar et al⁴⁰ reported heavy colonization of *L. vannamei* hepatopancreas with *V. parahaemolyticus*. Our results on the distribution of *V. parahaemolyticus* in shrimp aquaculture farms suggest that *V. parahaemolyticus* is commonly associated with farmed *L. vannamei*, farm water, and sediments.

In our study, the *Vibrio* counts in animal tissues (muscle and hepatopancreas) were in the range of 2-4 log CFU/g, while the counts in the sediment samples were uniformly around 6-7 log CFU/g (Table 3). In shrimp farm water samples, the counts ranged from 2.48-3.59 log CFU/ml. The water temperature and salinity of ponds sampled in this study did not vary significantly throughout the study period of three months. The salinity was in the range of 22-23 ppt, and the temperature was in the range of 27-29°C (Table 3). Therefore, salinity and temperature presumably did not influence *Vibrio* populations of shrimp ponds investigated in this study. In general, the *Vibrio* counts of the sediment samples were higher than the counts in tissues or the water samples (Table 3). The higher surface area of sediment particles and the associated nutrients might support higher bacteria populations in pond sediments compared

to water. In our study, the counts from the shrimp sample were higher than counts from the farm environment. Higher *Vibrio* counts in sediments compared to the water have been reported by other investigators^{41,42}. In an elegant study mimicking shrimp farm environment, Zheng et al⁴³ showed significant dissimilarities in microbial composition and diversity of water, sediment, and shrimp intestine. The microbial communities varied during different stages of shrimp culture. However, the changes in shrimp gut microbial communities did not resemble the changes in the surrounding environment⁴³. Differences in water, sediment, and shrimp gut *Vibrio* counts have been reported by several investigators⁴⁴⁻⁴⁶. While physicochemical parameters such as temperature, salinity, pH, and nutrients greatly influence *Vibrio* counts in the shrimp farm water and sediment, additional factors such as the health and immunity of shrimp, diet, pond application of probiotics, etc. determine the gut microbial communities of farmed shrimp⁴⁶.

Molecular characterization of *V. parahaemolyticus*

The main goal of this study was to understand the prevalence of human pathogenic *V. parahaemolyticus* in *P. vannamei* farm environment. The *toxR* positive isolates were further screened for the virulence genes *trh* and *tdh*. None of the isolates harbored the *tdh* gene, while the *trh* gene was detected in 2 isolates from shrimp hepatopancreas and 1 isolate from the pond sediment (Fig. 1 C). These isolates corresponded to two separate samples of shrimp and sediment. Thus, the incidence of *trh*⁺ *V. parahaemolyticus* in this study was 22.2% of shrimp samples and 16.6% of sediment samples. The *tdh* gene, which encodes a thermostable

Table 3. Total *Vibrio* counts of the samples analyzed from the shrimp farms

Date	Salinity (ppm)	Temp. (°C)	Shrimp (log CFU/g or ml) (n=9)			Sediment (log CFU/g) (n=6)	Water (log CFU/ml) (n=6)
			Tissue	Hepatopancreas	Hemolymph		
11-08-2017	23	27	4.09	5.13	<1 [¥]	6.33	2.4
21-08-2017	22	28	6.1	6.39	<1	7.08	2.28
11-09-2017	23	29	7.37	7.61	<1	7.67	3.53
22-09-2017	23	28	6.84	7.7	<1	7.44	3.66
12-10-2017	22	27	5.89	5.17	<1	6.42	3.25
26-10-2017	22	28	6.22	6.35	<1	7.48	3.47

n= number of samples; ¥ =Indicates no viable growth on plates by spread plate method.

direct hemolysin, is predominantly associated with clinical isolates of *V. parahaemolyticus* and only a small proportion (<1%) of the environmental isolates carry this gene^{5,6,47}. However, the incidence of *trh*-positive *V. parahaemolyticus* in wild-caught fish and shellfish is relatively higher than reported in this study. Ayyapan et al¹¹ isolated *trh*-positive *V. parahaemolyticus* from 2% of shellfish, 16% of coastal water, and 5% of coastal sediment samples, while Deepanjali et al⁶ reported that 59% of oyster samples harbored *trh*-positive *V. parahaemolyticus*. The reported prevalence of pathogenic *V. parahaemolyticus* in shrimp culture environment is low in India^{8,29,40}. However, recently a study from India reported a high prevalence of *tdh*- and *trh*-positive *V. parahaemolyticus* in shrimp farms³⁴. No or low prevalence of pathogenic *V. parahaemolyticus* isolated from aquaculture farms has been reported from Mexico⁴⁸, Brazil⁴⁹, Bangladesh³⁶, China⁵⁰, and Sri Lanka⁵¹. A study from Thailand on farmed shrimp reported a relatively higher (15.9% *tdh*⁺ and 4.6% *trh*⁺) prevalence of pathogenic *V. parahaemolyticus*⁵². In this study, we did not perform selective enrichment of the samples before the isolation of *V. parahaemolyticus* on TCBS agar. The total *Vibrio* count in the selective plates was as high as 7 log CFU/g in the hepatopancreas and pond sediment. Despite this high number, the *tdh*-positive *V. parahaemolyticus* was distinctly absent from the samples. It is worthwhile to compare direct plating with selective enrichment followed by isolation on selective agar, particularly in the case of farmed shrimp where high *V. parahaemolyticus* loads are expected, to determine if enrichment allows better isolation of *tdh*⁺ *V. parahaemolyticus*.

CONCLUSION

Risk assessment of *V. parahaemolyticus* in farmed shrimp requires continuous monitoring for total and pathogenic strains in the farm environment. The present study suggests that *V. parahaemolyticus* can be commonly found in farmed shrimp, both on the surface as well as in the hepatopancreas. However, *V. parahaemolyticus* encountered in shrimp farm environments in this study were, by far, non-pathogenic, although a small percentage of strains harbored the *trh* gene. Since *V. parahaemolyticus* is a known shrimp pathogen, the abundance of this bacterium

also depends on the health of the animals and physico-chemical characters of pond water. Pre-harvest testing of shrimp for pathogenic *V. parahaemolyticus* can help in ensuring the safety of seafood and compliance with the regulatory standards of importing countries.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

VK performed the bench work and sample analysis. SK and BBN conceptualized the project, supervised the research and analyzed the results. AKB and RV supervised the research and analyzed the results. All authors contributed to manuscript preparation, correction and approved the final version.

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None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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