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Strain variability and pathogenic potential of *tdh*-carrying *Vibrio parahaemolyticus* isolated from diseased *Penaeus monodon*

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ABSTRACT: A disease outbreak in 42-d-old black tiger shrimp *Penaeus monodon* juveniles from a commercial aquaculture farm in Kerala, India, was investigated. The cause of the disease outbreak was confirmed as *Vibrio parahaemolyticus* by biochemical tests, PCR targeting the *toxR* gene and pathogenicity testing of the isolates. All of the isolates tested negative by PCR specific for *V. parahaemolyticus* associated with acute hepatopancreatic necrosis disease (AHPND), implicating vibriosis unrelated to AHPND as the cause of mortality. Among the 19 isolates obtained, 2 possessed the *tdh* gene (coding for thermo-stable hemolysin), whereas none of the isolates possessed *trh*. The LD_{50} value of 8 isolates of *V. parahaemolyticus* from diseased and apparently healthy shrimp ranged from 2.7×10^4 to 4.9×10^5 CFU ml^{-1} by immersion challenge of *P. monodon* postlarvae. BOX-PCR and dendrogram analysis of the bacterial isolates revealed that the isolates from moribund and apparently healthy shrimp formed separate clusters, indicating that these isolates originate from separate clones. The isolates from moribund shrimp including *tdh*-positive *V. parahaemolyticus* clustered together. The present study represents the first report of *tdh*-positive *V. parahaemolyticus* causing disease in a shrimp farm.

KEY WORDS: *Penaeus monodon* · Tiger shrimp · Vibriosis · *Vibrio parahaemolyticus* · Hemolysin gene · Molecular typing · BOX PCR · Aquaculture

1. INTRODUCTION

Infectious diseases due to bacterial and viral pathogens are the major cause of economic losses in shrimp farming (Lavilla-Pitogo et al. 1998). Vibrionaceae is an important group of bacteria that is associated with infections in both larvae and juvenile shrimp (Chen et al. 2000, Joseph et al. 2015) and is present in the shrimp aquaculture environment as part of the normal microflora, residing on the cuticle or colonizing the gut or hepatopancreas (Brock & Lightner 1990).

Vibrio parahaemolyticus normally present in the aquatic environment at a low concentration can infect and cause disease outbreaks only when environmental factors favour rapid multiplication (Johnson et al. 2010). *V. parahaemolyticus* causes muscle necrosis, opacity, anorexia, slower growth and mortality in shrimp farms and hatcheries (Aguirre-Guzmán et al. 2010). Pathogenicity associated with *V. parahaemolyticus* varies with stage of physiological development of the shrimp, environmental stress, time and route of infection strain and dose of the pathogen (Karunasagar et al. 2005).

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Genetic analysis of *V. parahaemolyticus* isolates of environmental and clinical origins using various techniques indicate a high degree of clonality (Theethakaew et al. 2013, Urmersbach et al. 2014). However, isolates of *V. parahaemolyticus* that produce a plasmid-encoded toxin have been found to be the causative agent of acute hepatopancreatic necrosis syndrome/early mortality syndrome (AHPNS/EMS) and led to substantial losses in shrimp farms in China, Vietnam, Thailand and Malaysia (FAO 2013).

Recently, a disease outbreak in a commercial black tiger shrimp *Penaeus monodon* farm in Kerala, India, resulted in more than 25% mortality by the 42nd day of culture. The present study was intended to identify and characterize the pathogenic microorganism responsible for infection in diseased *P. monodon.*

2. MATERIALS AND METHODS

Diseased and apparently healthy shrimp samples were collected from an aquaculture farm where a disease outbreak was reported.

The infected shrimp samples were tested for viral pathogens such as hepatopancreatic parvovirus (HPV), monodon baculovirus (MBV) and infectious hypodermal and haematopoietic necrosis virus (IHHNV) using the World Organisation for Animal Health protocol (OIE 2016) and white spot syndrome virus (WSSV), yellow head virus (YHV) and Taura syndrome virus (TSV) using IQ2000 virus detection kits. The direct swab from the ulcers and the homogenized muscle tissue from the infected shrimp were directly plated onto tryptic soy agar (TSA, Difco) and thiosulfate–citrate–bile sucrose agar (TCBS, Difco) and incubated at 28°C overnight. Colonies were purified and the isolates were identified by biochemical tests (Garrity 2005). All the media used for the isolation were supplemented with and without 3% NaCl. Bacterial crude DNA was extracted from the *Vibrio parahaemolyticus* isolates as described by Cabrera-García et al. (2004).

The presence of *toxR*, *trh* and *tdh* genes in *V. parahaemolyticus* isolates was detected by PCR. *tdh*-positive (ATCC 33846) and *trh*-positive (ATCC 17802) *V. parahaemolyticus* were used as positive controls. The details of the primers used for the amplification of *toxR*, *tdh* and *trh* genes are given in Table 1. The PCR reaction was performed with 10× buffer (Thermo Fisher Scientific), 200 µM dNTPs, 25 pmol of each primer and 1.5 U of *Taq* polymerase (Thermo Fisher Scientific) in a Veriti Thermal Cycler (Applied Biosystems). The amplicons were separated on 1.5% agarose gels and documented by a gel documentation system (G:BOX F3, Syngene).

AP4 PCR assay was employed to determine the presence of AHPND-causing strain of *V. parahaemolyticus* (VP_{AHPND}) toxin gene as described by Dangtip et al. (2015). The amplicons were analysed in 1.5% agarose gel and documented on a UV transilluminator.

Experimental infections were performed on *Penaeus monodon* postlarvae with 4 isolates from moribund shrimp and 4 from apparently healthy shrimp. Prior to challenge, the experimental animals were tested for the presence of WSSV, MBV, HPV, IHHNV, YHV and TSV. The infectivity test was conducted by bath immersion challenge as described by Tran et al. (2013) in duplicate with test and control groups comprising 30 *P. monodon* postlarvae (PL20, ≤0.025 g). The postlarvae were immersed in different concentrations (1.3×10^3 to 1.7×10^6 CFU ml^{-1}) of bacteria for 15 min before transferring to 5 l of 20 ppt sterile seawater. Mortality was recorded daily for 5 d. The bacterial strains were reisolated and identified in moribund shrimp to confirm Koch's postulates. The LD_{50} was calculated by the method described by Reed & Muench (1938).

Table 1. Nucleotide sequences of the primers used in the study

Primer	Sequence (5′–3′)	Amplicon size (bp)	Reference
toxR F	GTC TTC TGA CGC AAT CGT TG	367	Kim et al. (1999)
toxR R	ATA CGA GTG GTT GCT GTC ATG		
tdh F	CCA CTA CCA CTC TCA TAT GC	251	Tada et al. (1992)
tdh R	GGT ACT AAA TGG CTG ACA TC		
trh F	GGC TCA AAA TGG TTA AGC G	250	Tada et al. (1992)
trh R	CAT TTC CGC TCT CAT ATG C		
AP4 F1	TGA GTA ACA ATA TAA AAC ATG AAA C	1269	Dangtip et al. (2015)
AP4 R1	ACG ATT TCG ACG TTC CCC AA		
AP4 F2	TTG AGA ATA CGG GAC GTG GG	230	
AP4 R2	GTT AGT CAT GTG AGC ACC TTC		
BOXA1R	CTA CGG CAA GGC GAC GCT	–	Versalovic et al. (1994)

BOX-PCR of the 8 *V. parahaemolyticus* isolates used for challenge experiment was performed as described by Versalovic et al. (1994). The PCR amplification reactions were carried out with 1.0 µl of template DNA, 10× PCR buffer, 1.5 mM $MgCl_2$, 1 U of *Taq* DNA polymerase (Thermo Scientific, USA), 200 µM dNTP and 1 µM of primer, with the following conditions: initial denaturation at 95°C for 2 min; 35 cycles of 94°C for 30 s, 43°C for 1 min, and 65°C for 3 min; and a final extension at 65°C for 8 min. Fingerprints of the bacterial isolates were analysed using GelCompar II software (Applied Maths) for cluster analysis via Pearson's coefficient and unweighted pair group mathematical averaging (UPGMA). The cophenetic correlation coefficient analysis was used to analyse the robustness of the dendrogram.

3. RESULTS

Based on the severity of infection, affected *P. monodon* shrimp presented clinical signs, including lethargy, emp ty gut, ulcers on the carapace, reddening of the body and pale hepatopancreas with discoloration. The infected shrimp samples were negative for the viral pathogens WSSV, HPV, MBV, IHHNV, YHV and TSV (Fig. 1). Circular (3 to 4 mm diameter), mucoid, green colonies were obtained on TCBS agar inoculated with tissue from infected and apparently healthy shrimp. Based on biochemical tests as described in Garrity (2005), all the isolates were tentatively identified as *V. parahaemolyticus*.

All 19 isolates from shrimp were confirmed as *V. parahaemolyticus* based on the amplification of the species-specific *toxR* region by PCR. Two of the isolates, INF3 and INF4, were found to carry the *tdh* gene, as revealed by amplification of the 251-bp product. None of the isolates carried the *trh* gene (Fig. 2).

All of the isolates were negative by both first and nested PCR assay for the detection of VP_{AHPND}, indicating that the disease in the farm was due to vibriosis rather than to AHPND.

The LD_{50} values of the isolates from both moribund and apparently healthy shrimp for *P. monodon* postlarvae (PL20) were similar and ranged from 2.7×10^4 to 4.9×10^5 CFU ml^{-1} (Table 2). Pure cultures of bacterial colonies re-isolated from diseased postlarvae after bacterial challenge were identified as the same species.

The fingerprints of *V. parahaemolyticus* isolates consisted of 4 to 12 amplification bands, ranging in size from 150 to 2750 bp. Each isolate exhibited a dif-

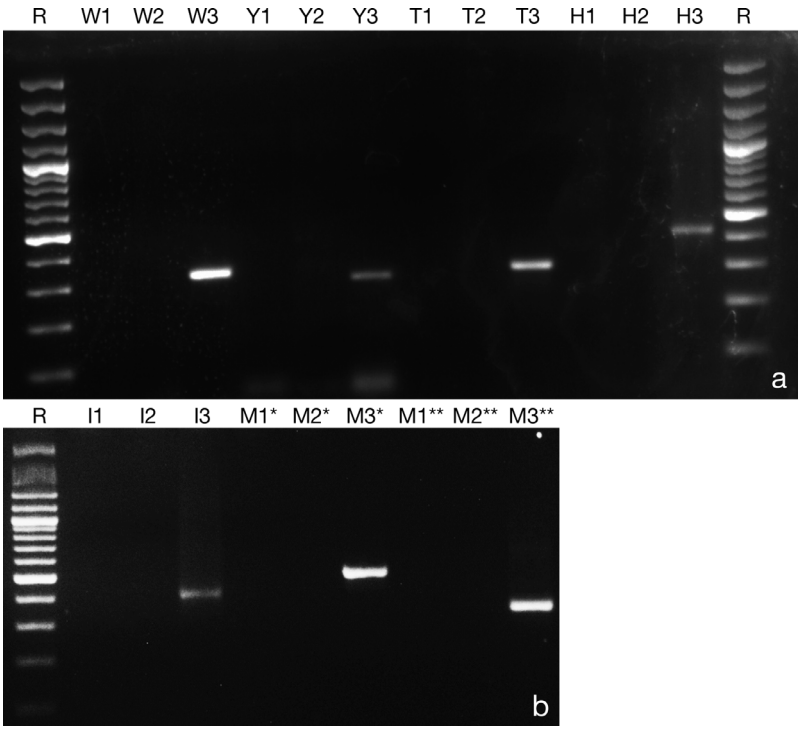

Fig. 1. Detection of viruses in shrimp samples by PCR. (a) White spot syndrome virus (WSSV), yellow head virus (YHV), Taura syndrome virus (TSV) and hepatopancreatic parvovirus (HPV); (b) infectious hypodermal and haematopoietic necrosis virus (IHHNV) and monodon baculovirus (MBV). 1: test sample; 2: negative control; 3: positive control; R: 100 bp plus DNA marker. *: results of first-step PCR; **: results of nested PCR; all other viruses were detected by single-step PCR

Table 2. LD_{50} value of *Vibrio parahaemolyticus* in *Penaeus monodon* postlarvae

Serial no.	Isolate	Source	LD_{50} (CFU ml^{-1})
1	LD1	Directly from lesion	2.7×10^4
2	INF2	From diseased shrimp	3.9×10^5
3	INF3		1.24×10^5
4	INF4		5.06×10^4
5	UINF2	From apparently healthy shrimp	8.6×10^4
6	UINF3		2.5×10^5
7	UINF4		4.9×10^5
8	UINF5		1.2×10^5

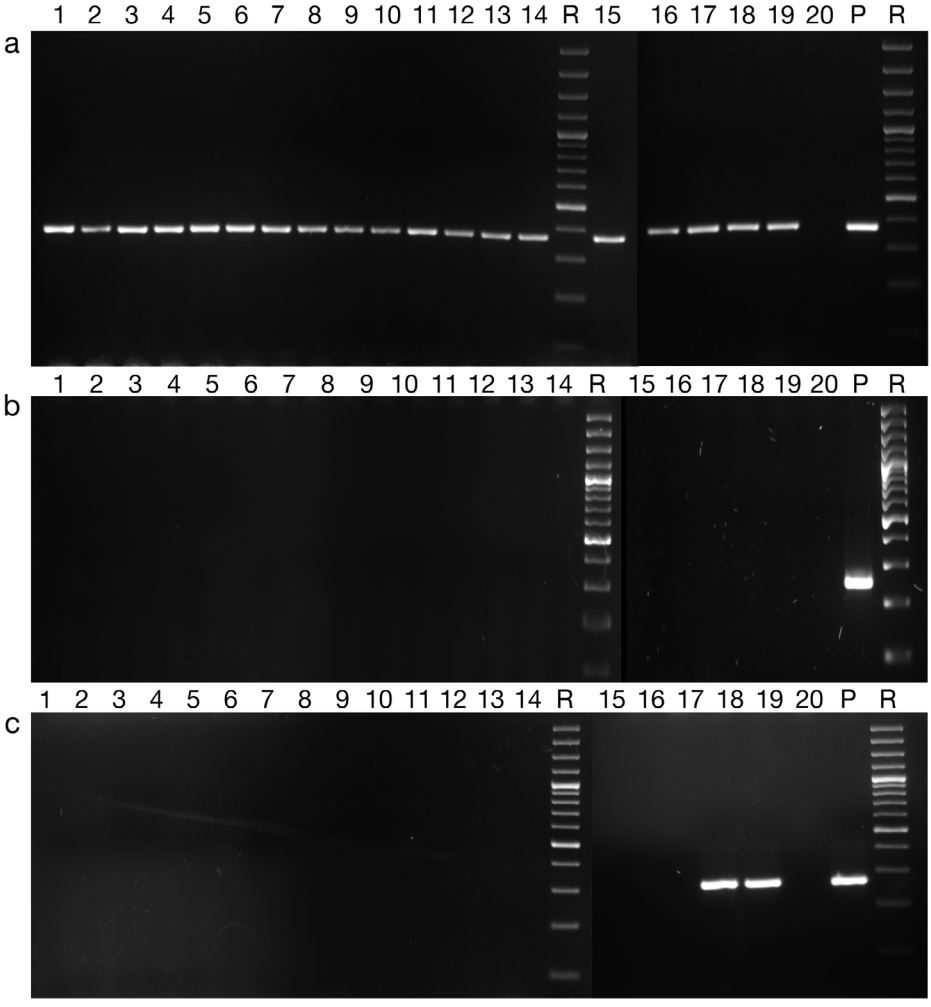

Fig. 2. Presence of (a) *toxR*, (b) *trh* and (c) *tdh* genes in *Vibrio parahaemolyticus* isolates. R: 100-bp Plus DNA marker; Lane P: *V. parahaemolyticus* ATCC 17802; Lane 20: negative PCR control; Lanes 1–19: isolates from diseased and apparently healthy shrimp (Lanes 18 and 19 are *V. parahaemolyticus* INF3 and *V. parahaemolyticus* INF4, respectively). The smear and/or extra band in the positive controls and in Lanes 18 and 19 in (c) is due to excess template DNA

ferent BOX profile, although a common band was observed in all 8 isolates at around 280 bp. Analysis of the similarity among the different profiles with the GelCompar II software identified 3 genomic clusters: the first group included isolates from diseased shrimp, the second cluster consisted of isolates from live healthy shrimp, and the reference isolate ATCC 17802 formed a separate cluster (Fig. 3).

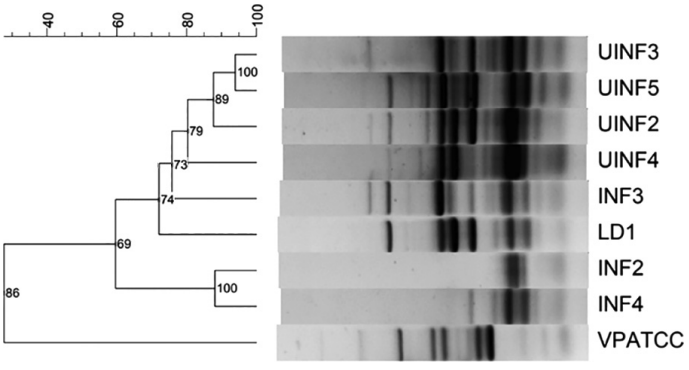

Fig. 3. BOX PCR fingerprinting of *Vibrio parahaemolyticus* isolated from *Penaeus monodon*. VPATCC: *V. parahaemolyticus* ATCC 17802; UNIF2, UNIF3, UNIF4 and UNIF5: isolates from apparently healthy shrimp; LD1: isolate directly from the lesion; INF2, INF3 and INF4: isolates from diseased shrimp

4. DISCUSSION

In the present study, we identified the presence of pathogenic *Vibrio parahaemolyticus* associated with a disease outbreak in a commercial shrimp farm in Kerala, India. All isolates from the outbreak were positive for the *V. parahaemolyticus*-specific *toxR* gene

(Croci et al. 2007). However, 2 isolates also possessed the thermostable direct hemolysin (*tdh*) gene, which is commonly used to determine the virulence of *V. parahaemolyticus* isolates in humans (Honda & Iida 1993). This is the first report of *tdh*-positive *V. parahaemolyticus* causing mortality in *Penaeus monodon.* Eight isolates, 4 each from moribund and apparently healthy shrimp, were chosen to determine the lethal dose in *P. monodon* postlarvae. The pathogenic potential of the isolates from both moribund and apparently healthy shrimp were highly similar, with LD_{50} values ranging from 2.7×10^4 to 4.9×10^5 CFU ml^{-1}.

The DNA-based typing of the bacterial isolates is useful for epidemiological models and for establishing causal relationships. BOX-PCR is an effective fingerprinting tool as compared to ERIC- and RAPD-PCR to determine the origin and divergence of *V. parahaemolyticus* strains from environmental samples (Yoke-Kqueen et al. 2013). Using BOX-PCR profiles, isolates from the present study were differentiated into clusters by UPGMA and the Pearson coefficient. The dendrogram derived from BOX-PCR analysis supported the contention that the isolates from moribund shrimp represented a cluster distinct from the isolates from apparently healthy shrimp, suggesting that the virulent group of isolates from the moribund shrimp might have originated from the same clone. The 2 isolates that carried the *tdh* gene belonged to 2 different subclusters, indicating that the isolates are genetically heterogeneous. Genetic analysis of *V. parahaemolyticus* from infected shrimp is limited, but is necessary to understand the disease characteristics in shrimp, which will help in developing new control strategies for diseases caused by *Vibrio* spp. The present study is the first report of infection caused by *tdh*-positive isolates of *V. parahaemolyticus* from India in cultured *P. monodon*. According to Yingkajorn et al. (2014), *tdh*-positive *V. parahaemolyticus* is prevalent in about 15.9% of the cultured shrimp in Thailand. The rapid replication of the *tdh*-positive *V. parahaemolyticus* during the disease outbreaks in non-specific hosts results in pathogen coming in contact with humans, increasing the risk of infection to humans.

The results of the present study reveal that highly pathogenic strains of *V. parahaemolyticus* are emerging and continue to cause lethality in farmed shrimp. This study has validated the pathogenic potential of the *V. parahaemolyticus* isolates in *P. monodon* postlarvae. The existence of *tdh*-positive *V. parahaemolyticus* in the aquaculture environment represents a significant threat to human health. Disease surveillance strategies and epidemiological studies of shrimp diseases are important to bring about effective control measures to reduce the risk infection by pathogenic *V. parahaemolyticus* and to guarantee the safety of seafood.

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Ethical Statement. In India, as per Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), it is not necessary to obtain ethical clearance to use shrimp for experimental research purposes.

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