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Research Note

Triplex-PCR Assay for the Simultaneous Detection and Differentiation of *Vibrio parahaemolyticus* in Farmed Shrimp

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Vibrio parahaemolyticus is an important bacterial pathogen affecting both human health and aquatic animal health. Initially, a multiplex PCR employing four sets of primers targeting *Vibrio* genus-specific regions and *V. parahaemolyticus* species-specific regions of the genome was optimized using a gradient PCR. The amplicons sizes for *Vibrio* genus detection were 663 bp (16S rRNA) and 689 bp (genus specific region of genome), while the amplicon sizes for *V. parahaemolyticus* species detection were 450 bp (*tlh* gene) and 297 bp (species-specific region of the genome). PCR conditions were first optimized using a *V. parahaemolyticus* type culture (ATCC 17802) and later used for testing *Vibrio* isolates from apparently healthy *Penaeus vannamei* shrimp and water samples from shrimp ponds. However, the use of only one set of primers targeting 16S rRNA was sufficient for detecting the *Vibrio* at the genus level, hence the multiplex-PCR was modified into a triplex PCR (663 bp, 450 bp and 297 bp amplicons). This triplex PCR finds application not only in screening shrimp samples, both during the aquaculture phase and in processed shrimp products, but also in screening aquaculture environment samples for the presence of *V. parahaemolyticus*.

Keywords: Multiplex-PCR, aquaculture, *Penaeus vannamei*, shrimp pathogens, food safety

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Vibrio parahaemolyticus is a gram-negative bacterium that causes gastroenteritis, wound infections, and septicaemia in humans, with the consumption of undercooked fishery products considered an important source of transmission (Letchumanan, Chan, & Lee, 2014; Ramachandran & Raymond, 2019). In shrimp aquaculture system, *V. parahaemolyticus* acts as an opportunistic pathogen that has been reported to be associated with Acute hepatopancreatic necrosis disease (AHPND), White faeces syndrome (WFS), or as co-infectious agent (Tran et al., 2013; Navaneeth et al., 2020; Caro et al., 2021; Valente & Wan, 2021; Zhang et al., 2021) causing significant economic distress. Several multiplex-PCR methods have been developed for the detection of *V. parahaemolyticus* alongside other common *Vibrio* pathogens (Bej et al., 1999, Tarr et al., 2007, Rao & Surendran, 2013; Kim, Ryu, Lee, Kim, & Kim, 2015).

Vibrio parahaemolyticus has implications for aquatic animal health during the aquaculture phase and for food safety in the post-harvest phase. The thermolabile haemolysin (*tlh*) gene is considered a species-specific marker for *V. parahaemolyticus* (Taniguchi, Hirano, Kubomura, Higashi, & Mizuguchi, 1986; Bej et al., 1999; Jones et al., 2012), and molecular methods based on the *tlh* gene are prescribed for identifying this bacterium in fish and shellfish matrices (USFDA, 2004). However, Klein, West, Mejia, and Lovell (2014) observed that no virulence factor gene sequence can be used for species level detection of *V. parahaemolyticus*, noting false positives with *tlh* gene-based identification (Croci et al., 2007). In this context, the present study describes a multiplex-PCR assay employing four sets of primers, i.e., two sets for detecting the *Vibrio* genus-

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specific regions and two sets for detecting *V. parahaemolyticus*-specific regions of the genome, to enable unambiguous identification of this pertinent human and aquatic animal pathogen. Based on the results, the multiplex was finally modified into triplex-PCR by using only three sets of primers.

Vibrio parahaemolyticus (ATCC 17802) was used for the PCR optimization. A total of 93 *Vibrio* isolates from apparently healthy farmed shrimp (n= 46) and shrimp-pond water (n=47) collected from shrimp farms (n=47) located in the East Godavari and West Godavari districts of Andhra Pradesh, India, were tested (Supplementary Fig. 1). All the *Vibrio* spp. were initially isolated by spread plating on Thiosulfate Citrate Bile salts Sucrose (TCBS) agar (Himedia, India). The sucrose non-fermenting vibrios were purified, biochemically identified (USFDA, 2004), and screened using the multiplex PCR assay targeting genes coding for thermolabile haemolysin, 16S rRNA, Recombinase A and hypothetical protein-VPA1095 (Table 1). A single bacterium from each shrimp or water sample was used for analysis, following the standard operating procedure (SOP) of the Network Programme on Antimicrobial Resistance in Fisheries (ICAR-NBFGR, 2019).

A 25 μL PCR mix was prepared by mixing 12.5 μL of PCR master mix (Takara Bio Inc., Japan), 0.75 μL each of the four sets of forward (10 μM) and reverse (10 μM) primers (Table 1) targeting *Vibrio* genus-specific regions (Tarr et al., 2007; Kim et al., 2015) and *V. parahaemolyticus* species-specific regions (Bej et al., 1999; Kim et al., 2015), 5.5 μL of nuclease free water and 1 μL of DNA template.

The PCR was performed in a thermal cycler (C1000 Touch, Bio-Rad) with an initial denaturation of 95 °C for 4 min, followed by 25 cycles of denaturation at 94 °C for 1 min, annealing at different temperatures ranging from 53 °C to 60 °C for 1 min, and extension at 72 °C for 1 min followed by final extension at 72 °C for 10 min. Eight different annealing temperatures were tested in the Gradient-PCR viz., 60, 59.5, 58.7, 57.4, 55.8, 54.6, 53.7, and 53 °C.

The multiplex PCR was carried out with an initial denaturation at 95 °C for 4 min, followed by 25 cycles of denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min, and extension at 72 °C for 1 min followed by final extension at 72 °C for 10 min.

The sensitivity of the multiplex PCR was tested using crude DNA extracted from serially diluted *V. parahaemolyticus* cells ranging from 3 to 3 x 10^7 cfu mL^{-1} as DNA template. Additionally, DNA extracted from 1 mL of overnight *V. parahaemolyticus* culture was serially diluted from 1620 μg mL^{-1} to 0.16 ng mL^{-1} and used as template in the multiplex PCR.

Post amplification, all the PCR products were loaded on a 2% agarose gel containing ethidium bromide (1 μg mL^{-1}), submerged in Tris Acetate EDTA (TAE) buffer, electrophoresed at 70 V for 90 min, and visualized in Gel documentation system (E-BOX, Vilber, France).

A multiplex PCR was developed using primers previously validated for the genus (Tarr et al., 2007; Kim et al., 2015) and species (Bej et al., 1999; Kim et al., 2015) detection of *V. parahaemolyticus*. Although the *tlh* gene-based detection of *V. parahaemolyticus* has been recommended for its detection in fish and shellfish matrices (USFDA, 2004), certain inadequacies have been reported (Klein et al., 2014). Kim et al. (2015) described a detection method for *V. parahaemolyticus* targeting a sequence coding for a hypothetical protein VPA1095. The present multiplex-PCR method utilizes both these sets of primers to ensure unequivocal identification of *V. parahaemolyticus* isolated from shrimp and water samples from aquaculture ponds. Moreover, to differentiate other *Vibrio* spp. and to cross verify the genus, two additional sets of primers specific to the *Vibrio* genus targeting the 16S rRNA region (Tarr et al., 2007) and Recombinase A (Kim et al., 2015) were included in the multiplex PCR.

The annealing temperature for the different primers (Table 1) ranged between 57 °C and 60 °C. In order to ascertain the optimum annealing temperature, gradient PCR was performed with eight different annealing temperatures viz., 60, 59.5, 58.7, 57.4, 55.8, 54.6, 53.7, and 53 °C using the DNA template from *V. parahaemolyticus* (ATCC 17802) and a field isolate. The multiplex PCR yielded three bands viz., 297 bp (hypothetical protein) and 450 (thermolabile haemolysin) corresponding to the species-specific region of *V. parahaemolyticus,* and one band (663-689 bp) corresponding to the *Vibrio* genus-specific region. Although two sets of primers targeting the *Vibrio* genus-specific region were used, only a single band was seen due to similar amplicon sizes. Three clear amplification bands were observed at temperatures of 53, 53.7, 54.6, 55.8, and 57.4 °C, while light bands appeared at higher temperatures of 58.7, 59.5, and 60 °C (Fig. 1). For the *V. parahaemolyticus* type

Table 1. Target region, nucleotide sequence, amplicon size of the primers used in the multiplex-PCR for *Vibrio parahaemolyticus*

Category	Target region	Primer name	Primer sequence*	Annealing	Amplicon (bp)	Reference
Vibrio parahaemolyticus	*tlh* gene	L-TLH	5' – AAA GCG GAT TAT GCA GAA GCA CTG-3'	60 °C for 1 min, 25 cycles	450	Bej et al. (1999)
		R-TLH	5'–GCT ACT TTC TAG CAT TTT CTC TGC-3'			
	Hypothetical protein-VPA1095	VP-1155272-F	5'–AGC TTA TTG GCG GTT TCT GT CGG-3'	60 °C for 30 s, 25 cycles	297	Kim et al. (2015)
		VP-1155272-R	5'–CKC AAG ACC AAG AAA AGC CGT C-3'			
Vibrio genus	16S rRNA	*V*.16S-700F	5'- CGG TGA AAT GCG TAG AGA T-3'	57 °C for 1 min, 35 cycles	663	Tarr et al. (2007)
		V.16S-1325R	5'- TTA CTA GCG ATT CCG AGT TC-3'			
	Recombinase A	VG-C269435 2- F46	5'- GTC ARA TTG AAA ARC ART TYG GTA AAG G-3'	60 °C for 30 s, 25 cycles	689	Kim et al. (2015)
	VG-C269435 2-R734	5'-ACY TTR ATR CGN GTT TCR TTR CC- 3'				

where K = G + T; R = A + G; Y = C + T; N = A + C + G + T

culture, a faint band of 297 bp related to species specific amplification was found at higher annealing temperatures, whereas the genus-specific band was even lighter in field isolate (663-689 bp). Based on these results, 54 °C was chosen as optimal annealing temperature for proper amplification with the multiplex PCR.

Sucrose non-fermenting *Vibrio* isolates (n=46) obtained from *P. vannamei* shrimp collected across different aquaculture farms (one isolate per farm) were screened using the developed multiplex PCR. The results showed that all 46 isolates belonged to the genus *Vibrio,* and 25 of these were identified as *V. parahaemolyticus* (Fig. 2). Among the 25 positive isolates, only three isolates (wells 3, 22 and 23) exhibited relatively weak species-specific bands, while the remaining 22 isolates showed prominent genus- and species-specific bands. The *V. parahaemolyticus* isolates identified by the multiplex-PCR were further confirmed through biochemical identification tests such as sugar utilization (sucrose, cellobiose, lactose, arabinose, mannose, mannitol), amino acid decarboxylation (lysine, ornithine, arginine), salt tolerance (0, 3, 6, 8, 10%), oxidase, ONPG, and Voges-Proskauer tests (USFDA, 2004).

Similarly, sucrose non-fermenting *Vibrio* colonies isolated from water samples collected from shrimp aquaculture ponds (n=47) were screened using the

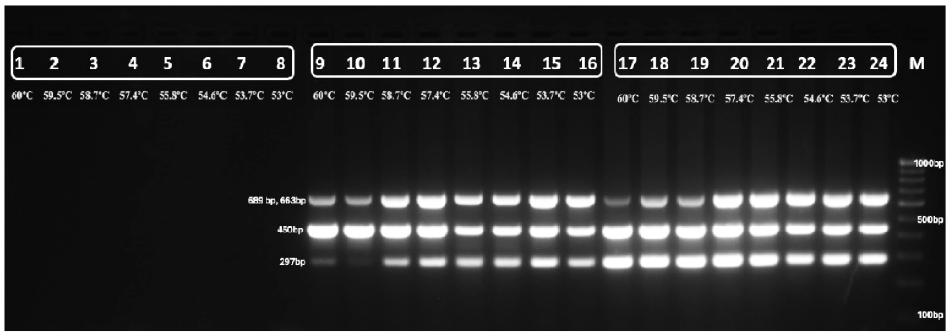

Fig. 1. Gradient-multiplex PCR optimization with different annealing temperatures for the detection of *Vibrio parahaemolyticus*

Lane M: 100bp ladder (Genei, India), lanes 1-8: Negative control, lanes 9-16: V. parahaemolyticus ATCC 17802, Lanes 17-24 V. parahaemolyticus field isolate (Gradient annealing temperatures: 60, 59.5, 58.7, 57.4, 55.8, 54.6, 53.7, and 53 °C)

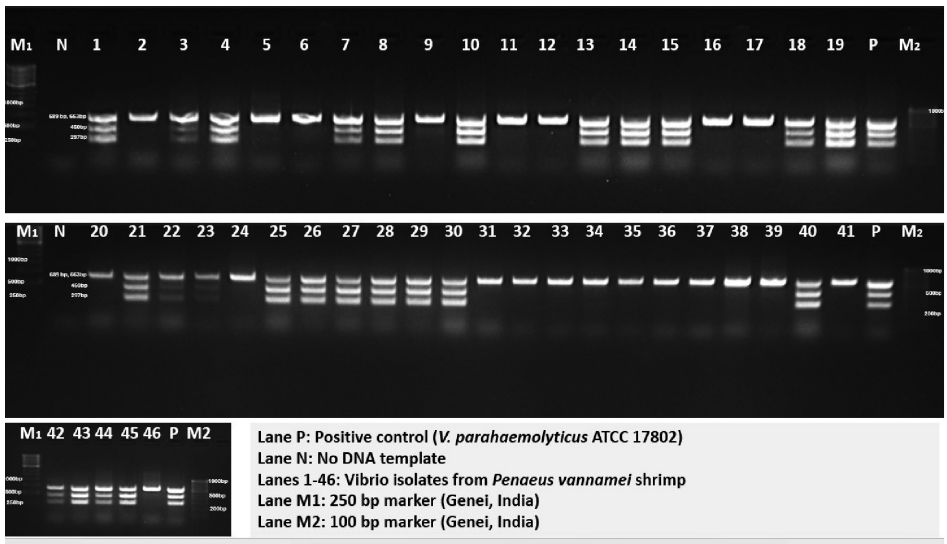

Fig. 2. Multiplex PCR for detection of *Vibrio parahaemolyticus* isolated from healthy farmed *Penaeus vannamei*

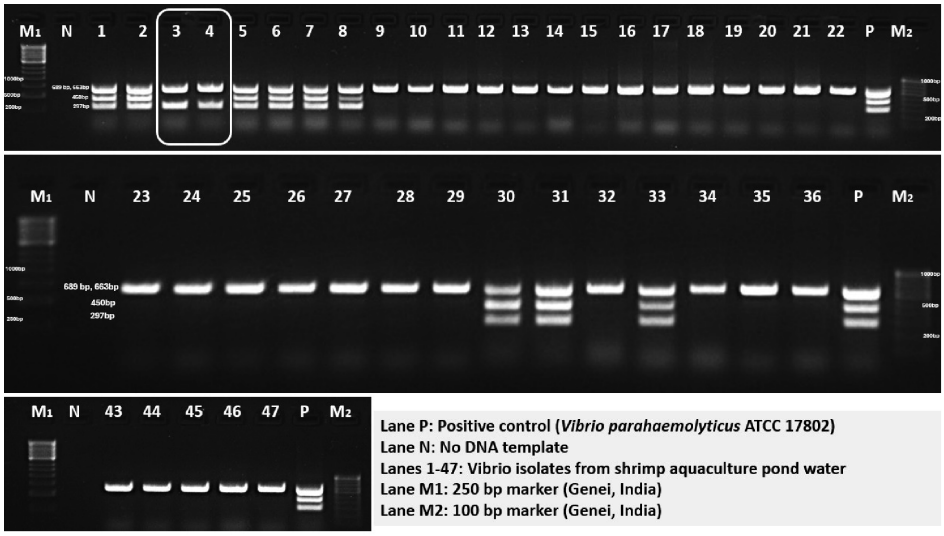

Fig. 3. Multiplex-PCR for the detection of *Vibrio parahaemolyticus* in shrimp aquaculture pond water (Inset box: *tlh* gene negative isolates)

developed multiplex PCR. The results revealed that 11 of the water samples were positive for *V. parahaemolyticus* (Fig. 3). Interestingly, two *V. parahaemolyticus* isolates (well 3 and well 4) did not show amplification of the *tlh* gene (450 bp), although the species-specific sequence (297 bp) targeting a hypothetical protein was amplified. This finding suggests that relying solely on *tlh* gene amplification for confirming *V. parahaemolyticus* may be inadequate. While previous studies have reported false positive associated with the *tlh* gene (Croci et al., 2007), this study reports the lack of *tlh* in few isolates of *V. parahaemolyticus*.

Crude DNA extracted from serially diluted *V. parahaemolyticus* cells, ranging between 3 cfu mL^{-1} to 3 x 10^7 cfu mL^{-1}, was used as the template for the multiplex PCR. The results indicated that the multiplex PCR could reliably detect *V. parahaemolyticus* at a concentration of 3 x 10^5 cfu mL^{-1} and above (Fig. 4a). Although the genus-specific amplicon was visible at 3 x 10^4 cfu mL^{-1}, the species-specific amplicons were absent at that concentration. Serial dilution of DNA (0.16 ng mL^{-1} to 1620 µg mL^{-1}) extracted from *V. parahaemolyticus* revealed that the multiplex PCR could detect DNA template as low as 1.62 µg mL^{-1} (Fig 4b).

Though the multiplex PCR utilized four sets of primers, only three bands were observed, as the amplicon sizes (663 and 689 bp) for the genus level

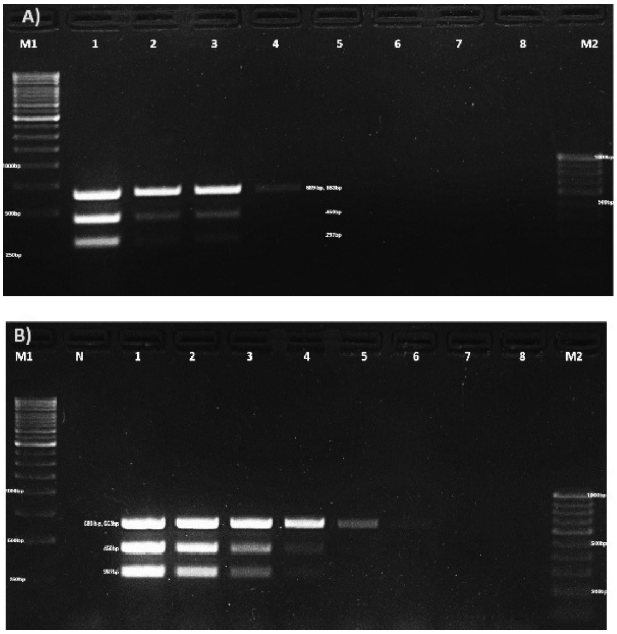

Fig. 4 Sensitivity of the multiplex-PCR for detecting *V. parahaemolyticus*

4A: Decreasing concentration of bacterial cells

Lane M1: 250bp ladder (Genei, India), lane 1: 3 x 10^7 cfu mL^{-1}, lane 2:3 x 10^6 cfu mL^{-1}, lane 3: 3 x 10^5 cfu mL^{-1}, lane 4: 30000 cfu mL^{-1}, lane 5: 3000 cfu mL^{-1}, lane 6: 300 cfu mL^{-1}, lane 7: 30 cfu mL^{-1}, lane 8: 3 cfu ml^{-1}, Lane M2: 100 bp ladder (Genei, India)

4B: Decreasing concentration of DNA template

Lane M1: 250 bp ladder (Genei, India), lane N: no DNA template, lane 1: 1620 µg mL^{-1}, lane 162 µg mL^{-1}, lane 3: 16.2 µg mL^{-1}, lane 4 : 1.62 µg mL^{-1}, lane 5: 0.16 µg mL^{-1}, lane 6: 16 ng mL^{-1}, lane 7: 1.6 ng mL^{-1}, lane 8: 0.16 ng mL^{-1}, lane M2: 100 bp ladder (Genei, India)

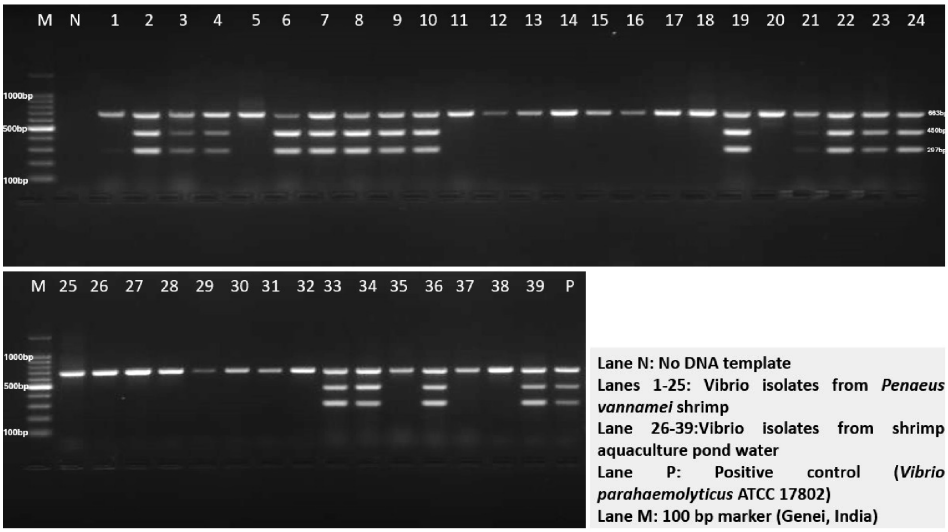

Fig. 5. Triplex PCR for the detection of Vibrio genus and *Vibrio parahaemolyticus* isolated from farmed shrimp and aquaculture pond water

detection were almost similar. Hence, the need for using two primer sets for detecting *Vibrio* bacteria at the genus level was revisited by performing two uniplex-PCRs viz., one targeting 16S rRNA (Tarr et al., 2007) and the second targeting Recombinase A (Kim et al., 2015). The results indicated that the primer set targeting 16S rRNA (Supplementary Fig. S2) was reliable and effective for identifying the bacteria as *Vibrio* at the genus level compared to the primer set targeting Recombinase A (Supplementary Fig. S3). Accordingly, the multiplex PCR was modified as a triplex PCR that included only three sets of primers namely V.16S-700F & V.16S-1325R primers (Tarr et al., 2007) for categorizing the bacteria as belonging to *Vibrio* genus and L-TLH & R-TLH primers (Bej et al., 1999), and VP- 1155272-F & VP-1155272-R primers (Kim et al., 2015) for species-specific detection of *V. parahaemolyticus*. The triplex-PCR was tested with presumptive *Vibrio* bacteria isolates from *P. vannamei* shrimp (n=25) and pond water samples (n=14). The triplex-PCR identified all the vibrios as belonging to *Vibrio* genus (100%) and 52% of the shrimp isolates and 28.5% of the pond water isolates as *V. parahaemolyticus* (Fig. 5). All *V. parahaemolyticus* isolates identified by the triplex-PCR were also confirmed as the same through biochemical tests (USFDA, 2004).

The triplex-PCR finds application in the detection and differentiation of *V. parahaemolyticus* isolated from farmed aquatic animals and environment samples. As this bacterium is harmful both to the aquatic animals and human beings, the triplex-PCR finds application in the aquaculture phase by the aquatic animal health laboratories and also during post-harvest by the food safety laboratories. Further, the triplex-PCR is advantageous in terms of cost, time, and physical effort. The cost of triplex-PCR assay for analysing seven samples and loading the PCR products on an 8-well agarose gel of 6.6 cm x 6.2 cm size was calculated to be INR 5221(~USD 60) lower than that of performing three separate uniplex PCRs. Moreover, the gel electrophoresis run time of triplex-PCR was 4.5 hours faster.

A triplex-PCR method was successfully developed for the detection of *V. parahaemolyticus,* employing two sets of primers targeting the species-specific sequences and one set of primers targeting the genus-specific region. The triplex-PCR finds application in screening sucrose non-fermenting colonies (green colonies) on TCBS agar in monitoring aquatic animal health, evaluating environmental quality in aquaculture systems, and ensuring post-harvest food safety.

References

Bej, A. K., Patterson, D. P., Brasher, C. W., Vickery, M. C. L., Jones, D. D., & Kaysner, C. A. (1999). Detection of total and hemolysin-producing *Vibrio parahaemolyticus* in shellfish using multiplex PCR amplification of *tl*, *tdh* and *trh*. *Journal of Microbiological Methods*, *36*(3), 215-225. https://doi.org/10.1016/S0167-7012(99)00037-8.

Caro, L. F. A., Mai, H. N., Cruz-Florez, R., Marcos, F. L. A., Alenton, R. R. R., & Dhar, A. K. (2021). Experimental reproduction of White Feces Syndrome in whiteleg shrimp, *Penaeus vannamei*. *PLoS One*, *16*(12), Article e0261289. https://doi.org/10.1371/journal.pone.0261289.

Croci, L., Suffredini, E., Cozzi, L., Toti, L., Ottaviani, D., Pruzzo, C., Serratore, P., Fischetti, R., Goffredo, E., Loffredo, G., Mioni, R., & the *Vibrio parahaemolyticus* Working Group (2007). Comparison of different biochemical and molecular methods for the identification of *Vibrio parahaemolyticus*. *Journal of Applied Microbiology*, *102*(1), 229–237. http://dx.doi.org/10.1111/j.1365-2672.2006.03046.x.

Jones, J. L., Ludeke, C. H. M., Bowers, J. C., Garrett, N., Fischer, M., Parsons, M. B, Bopp, C. A., & DePaola, A. (2012). Biochemical, serological, and virulence characterization of clinical and oyster *Vibrio parahaemolyticus* isolates. *Journal of Clinical Microbiology*, *50*(7), 2343–2352. https://doi.org/10.1128/jcm.00196-12.

ICAR-NBFGR. (2019). *Network programme on AMR in fisheries: Standard Operating procedures* (2nd ed.). ICAR-National Bureau of Fish Genetic Resources.

Kim, H. J., Ryu, J. O, Lee, S. Y., Kim. E. S., & Kim, H. Y. (2015). Multiplex PCR for detection of the *Vibrio* genus and five pathogenic *Vibrio* species with primer sets designed using comparative genomics. *BMC Microbiology*, *15*, Article 239. https://doi.org/10.1186/s12866-015-0577-3.

Klein, S. L., West, C. K. G, Mejia, D. M., & Lovell, C. R. (2014). Genes similar to the *Vibrio parahaemolyticus* virulence-related genes *tdh*, *tlh*, and *vscC2* occur in other Vibrionaceae species isolated from a pristine estuary. *Applied and Environmental Microbiology*, *80*, 595–602. https://doi.org/10.1128/AEM.02895-13.

Letchumanan, V., Chan, K. G., & Lee, L. H. (2014). *Vibrio parahaemolyticus*: a review on the pathogenesis, prevalence, and advance molecular identification techniques. *Frontiers in Microbiology*, *5*, Article 705. https://doi.org/10.3389/fmicb.2014.00705.

Navaneeth, K. A., Bhuvaneswari, T., Rajan, J. J. S., Alavandi, S. V., Vijayan, K. K., & Otta, S. K. (2020). Characterization of *Vibrio parahaemolyticus* isolates from shrimp farms of Southeast coast of India with special reference to Acute Hepatopancreatic Necrosis Disease (AHPND) status. *Aquaculture*, *518*, Article 734813. https://doi.org/10.1016/j.aquaculture.2019.734813.

Rao, B. M., & Surendran, P. K. (2013). Pathogenic vibrios in *Penaeus monodon* shrimp hatcheries and aquaculture farms. *Fishery Technology*, *50*(2), 161-167.

Ramachandran, A., & Raymond, A. (2019). Bacterial pathogens in seafood–Indian scenario. *Fishery Technology*, *56*(2), 1-22.

Tarr, C. L., Patel, J. S., Puhr, N. D., Sowers, E. G., Bopp, C. A., & Strockbine, N. A. (2007). Identification of *Vibrio* isolates by a multiplex PCR assay and *rpoB* sequence determination. *Journal of Clinical Microbiology*, *45*, 134–140. https://doi.org/10.1128/jcm.01544-06.

Taniguchi, H., Hirano, H., Kubomura, S., Higashi, K., & Mizuguchi, Y. (1986). Comparison of the nucleotide sequences of the genes for the thermolabile hemolysin from *Vibrio parahaemolyticus*. *Microbial Pathogenesis*, *1*(5), 425-432. https://doi.org/10.1016/0882-4010(86)90004-5.

Tran, L., Nunan, L., Redman, R. M., Mohney, L. L., Pantoja, C. R., Fitzsimmons, K., & Lightner, D. V. (2013). Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. *Diseases of Aquatic Organisms*, *105*, 45-55. https://doi.org/10.3354/dao02621.

U.S. Food & Drug Administration (USFDA). (2004). *Bacteriological Analytical Manual, Chapter 9: Vibrio*. Retrieved from https://www.fda.gov/food/laboratory-methods-food/bam-chapter-9-vibrio. Accessed October 25, 2024.

Valente, C. S., & Wan, A. H. L. (2021). Vibrio and major commercially important vibriosis diseases in decapod crustaceans. *Journal of Invertebrate Pathology*, *181*, Article 107527. https://doi.org/10.1016/j.jip.2020.107527.

Zhang, X., Sun, J., Han, Z., Chen, F., Lv, A., Hu, X., Sun, X., Qi, H., & Guo, Y. (2021). *Vibrio parahaemolyticus* alters the community composition and function of intestinal microbiota in Pacific white shrimp, *Penaeus vannamei*. *Aquaculture*, *544*, Article 737061. https://doi.org/10.1016/j.aquaculture.2021.737061.