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Original Article

Pathogenic Vibrios associated with loose shell syndrome in mangrove crabs (*Scylla* spp.)

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Abstract: An emerging disease known as loose shell syndrome, with unknown etiology, impacts mangrove crab aquaculture in the Philippines. This study investigated the presence and characterized pathogenic *Vibrio* spp., which might be implicated in loose shell syndrome in mangrove crabs, *Scylla* spp. Five bacterial isolates associated with loose shell syndrome in mangrove crabs were obtained and purified. Polymerase chain reaction (PCR) amplification using *Vibrio*-specific primers identified one isolate carrying the hemolysin (*vhh*) virulence gene of *Vibrio harveyi*, which was later confirmed by sequencing of the 16S rRNA. The other non-*Vibrio* isolates were *Proteus*, *Shewanella*, and *Stutzerimonas*. This study provides valuable insights into the possible etiology of loose shell syndrome in mangrove crabs, contributing to a better understanding of whether the condition stems from bacterial, environmental, or a combination of both factors.

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Introduction

Mangrove crabs (*Scylla* spp.) are among the top species cultured in the Philippines for aquaculture production, as they are well-liked for their taste, texture, and nutritive value (Triño et al., 1999). In fact, the country is the second-top producer of mangrove crabs in the world. As one of the major mangrove crab-producing countries, the Philippines needs to ensure the sustainability of the mangrove crab farming industry. However, this goal faces significant challenges due to the emergence of infectious and non-infectious diseases.

Loose shell syndrome is characterized by a loosely attached carapace, diminished internal mass, and reduced body size (Villarias et al., 2024). It shares similarities with the loose shell syndrome observed in shrimps, where the exoskeleton covering the abdominal musculature becomes weak, loose, or soft (AftabUddin et al., 2018). It also results in a noticeable reduction in body size and significantly diminished internal mass relative to typical specimens. A possible etiological agent for loose shell syndrome in mangrove crabs is the *Vibrio* species, which is known for causing bacterial diseases in aquaculture (Haseeb and Singh, 2012). These bacteria predominantly affect the gills, guts, hepatopancreas, hemolymph, and exoskeleton of crabs (Saha et al., 2023). *Vibrio* spp. have also been identified in shrimps with loose shell syndrome, raising the possibility of a similar link in mangrove crabs (Jayasree et al., 2008; Naik et al., 2020).

In a previous study, Villarias et al. (2024) found an association between the occurrence of Vibrios in mangrove crabs with loose shell syndrome, suggesting the potential role of this group of bacteria in the progression of the disease. To gain a more comprehensive understanding of how Vibrios contribute to the occurrence of loose shell syndrome in mangrove crabs, the present study aimed to characterize these pathogenic Vibrios at the morphological, biochemical, and molecular levels. The data derived from this study will provide crucial information on the transmission dynamics and host-pathogen interactions of these Vibrios in order to develop targeted interventions for effective health management of mangrove crab aquaculture.

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Table 1. Summary of primer pairs used to target pirA, vhh, and toxR genes for PCR amplification.

Primer name	Sequence	Expected product size	Gene	Reference
AP3-F	5'-ATGAGTAACAATATAAAACATGAAAC-3'	336 bp	*pir*A	Sirikharin et al. (2014)
AP3-R	5-GTGGTAATAGATTGTACAGAA -3'			
VhhF	5'-GCATTGGGTGACAGCTTGTCG-3'	320 bp	*vhh*	Castroverde et al. (2006)
VhhR	5'-CGGTTGTAGTTCATGAAGTCATTC-3'			

Materials and Methods

Sample collection and isolation of putative *Vibrio* spp.: Mangrove crabs that exhibit loose shell syndrome were purchased from a local crab landing site in Roxas City, Capiz, and transported to the laboratory of the National Institute for Molecular Biology and Biotechnology at the University of the Philippines Visayas for analysis. The affected crabs were identified by their soft, thin carapace, reduced body size, and reduced internal mass compared to apparently healthy crabs. The gills and gut were excised, weighed, and placed in a 1.5 mL centrifuge tube. An equal volume of normal saline solution (NSS) was added, and the samples were homogenized. Serial dilutions and the isolation of Vibrios and Vibrio-like colonies on Thiosulfate–Citrate–Bile Salts–Sucrose (TCBS) agar plates followed the method described previously (Villarias et al., 2024).

Biochemical characterization: Five bacterial isolates were further characterized after initial NA+ and TCBS agar plate assessments. Colony features were evaluated on Chromogenic Vibrio agar (CVA), and Gram staining further detailed their morphology. Biochemical properties were analyzed following American Society for Microbiology protocols, including tests for motility, hydrogen sulfide production, catalase, citrate utilization, and gelatin hydrolysis, based on Bergey's Manual (Holt et al., 2000).

Molecular identification of pathogenic Vibrios: DNA from the isolates was extracted using a commercial genomic DNA extraction kit (Invitrogen™ PureLink™ Genomic DNA Mini Kit) following the manufacturer's protocol. The extracted DNA was used to screen for the presence of pathogenic *Vibrio* spp. via polymerase chain reaction (PCR) using different primer sets, and portions of each isolate were sent for sequencing (Macrogen Inc., Korea). Different primer sets were utilized for the PCR analysis to detect the target virulent genes. One of the primers used was the VhhFR primer pair (Castroverde et al., 2006) for the specific amplification of a 320-bp fragment of the hemolysin gene from Philippine isolates of *Vibrio* spp. The AP3 primer set of Sirikharin et al. (2014) targeted the toxin gene pirA causing Acute Hepatopancreatic Necrosis Disease (AHPND). The primer pair is unique to the DNA sequences derived from the plasmid of *V. parahaemolyticus* and has been shown to effectively target *V. parahaemolyticus* AHPND-positive isolates (Kongrueng et al., 2015). Table 1 summarizes the primer pairs used in this study for PCR and their respective sequences, target genes, and expected product size.

Prior to loading the DNA samples on the thermal cycler, a PCR reaction mix was prepared, which includes DNA polymerase, dNTPs, PCR buffer, and primers following the procedures of Caipang et al. (2010). Amplification was performed with an initial denaturation of 95°C for 1 minute, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 53°C for 30 seconds, elongation at 72°C for 1 minute, and a final elongation at 72°C for 5 minutes completed the reaction. PCR products were resolved on a 1% agarose gel alongside the VC 100bp Plus DNA Ladder (Vivantis Technologies) for size determination. Gel images were analyzed using the Gel Doc XR+ System to identify isolates positive for the target genes.

Phylogenetic analysis: The 16S rRNA sequences of the five bacterial isolates were compared to the sequences available in the GenBank database using

Table 2. Morphological characterization of selected bacterial isolates from mangrove crabs with loose shell syndrome.

Isolate	Colony Morphology					Gram Stain
	Margin	Elevation	Color on NA^+	Color on TCBS	Color on CVA	
P1	Smooth	Convex	Creamy white	Green	Pink-rose	Negative
P4	Smooth	Convex	Creamy white	Yellow	Colorless	Negative
P5	Smooth	Convex	Creamy white	Green	Pink-rose	Negative
P6	Smooth	Convex	Creamy white	Green	Pink-rose	Negative
P10	Smooth	Convex	Creamy white	Yellow	Colorless	Negative

Table 3. Biochemical characterization of selected bacterial isolates from mangrove crab with loose shell syndrome.

Isolate	Motility	H_2S Production	Catalase	Gelatin Hydrolysis	Sucrose Fermentation	Citrate Utilization
P1	+	+	+	-	-	+
P4	+	-	+	+	+	-
P5	+	+	+	+	-	-
P6	+	+	+	+	+	-
P10	+	+	+	+	-	-

the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) to determine bacterial identities. Closely related species or strains with high similarity scores, as determined by BLASTn results, were selected, with *Acinetobacter baumannii* DSM30007 included as an outgroup for rooting the phylogenetic tree. Multiple sequence alignments were performed using MAFFT (Version 7) (Katoh et al., 2019), followed by trimming with TrimAl (Capella-Gutierrez et al. 2009) to remove regions with poor alignment and gaps. The trimmed alignments in FASTA format were then converted to PHYLIP file for tree-building using IQTree, and the constructed phylogenetic tree was visualized using FigTree (Version 19 1.4.4). The species closest to the clade containing the reference sequence was identified for each bacterial isolate.

Results and Discussions

The morphological characteristics of the five selected bacterial isolates are detailed in Table 2. All isolates were Gram-negative and produced smooth, convex, and creamy-white colonies on NA+. On TCBS, isolates P1, P5, and P6 produced green colonies, whereas isolates P4 and P10 produced yellow colonies or sucrose fermenters. On CVA, isolates P1, P5, and P6 produced pink rose colonies, while isolates P4 and P10 exhibited colorless colonies.

Additionally, microscopic examination revealed that isolate P2 exhibited the characteristic comma-shaped morphology of *Vibrio* species. Table 3 provides an overview of the biochemical properties of the bacterial isolates. All isolates are shown to have tested positive for motility and catalase test. However, in the citrate utilization test, only isolate P1 tested positive, while the remaining isolates tested negative. Conversely, in the gelatin hydrolysis test, isolate P1 tested negative, whereas the other isolates tested positive. The results from the agarose gel electrophoresis of the PCR showed that none of the isolates amplified the *pirA* gene as there is no distinct band that can be observed. This absence suggests the lack of virulent *V. parahaemolyticus* strains harboring the *pirA* gene among the tested isolates.

Figure 1 presents the gel electrophoresis result of the PCR aimed at detecting the *vhh* gene in the selected isolates. A distinct band can be observed above the 300-bp marker in lane 2 (Isolate P2), indicating the presence of the *vhh* gene. This suggests that Isolate P2 is likely a pathogenic *Vibrio*. The other

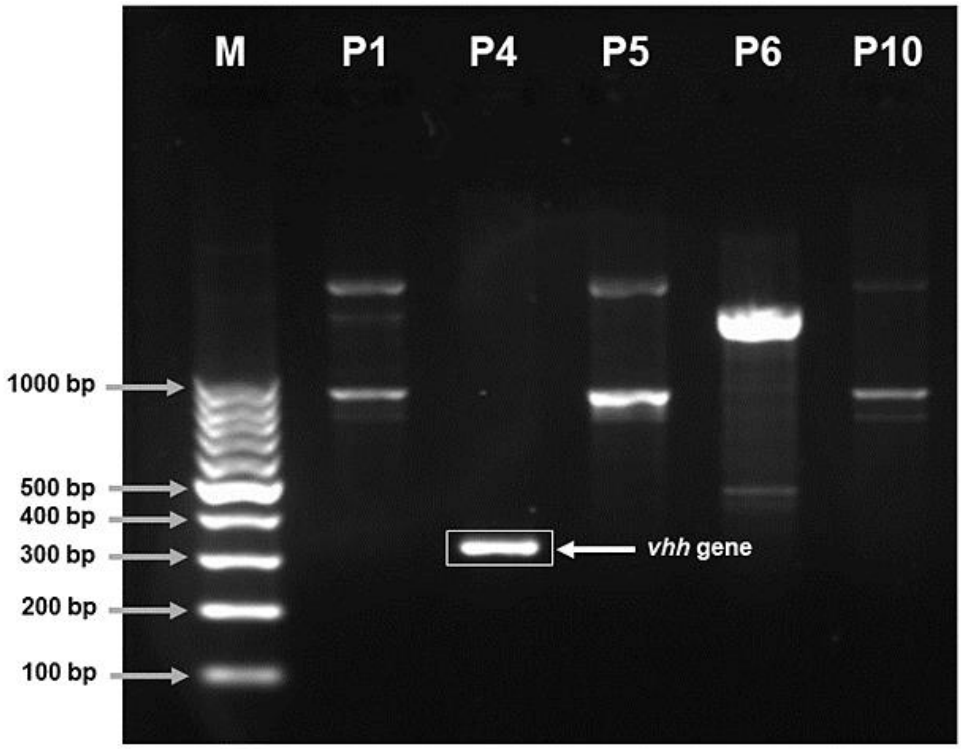

Figure 1. Agarose gel electrophoresis of PCR products targeting the *vhh* gene.

lanes show varying degrees of DNA fragments or band sizes, which may imply that the observed bands in these lanes result from non-specific amplification.

The phylogenetic tree, generated through maximum likelihood analysis of 16S rRNA sequences, is illustrated in Figure 2. The results indicate that isolate P4 forms a distinct clade with *Vibrio harveyi* and is supported by a bootstrap value of 94%, confirming its classification within the *Vibrio* genus. Conversely, isolates P1 and P5 cluster closely with *Shewanella* algae, with 73% and 88% bootstrap values, respectively. Isolate P6 shares significant sequence similarity with *Stutzerimonas stutzeri*, supported by a bootstrap value of 70%. Additionally, isolate P10 is closely related to *Proteus mirabilis*, with the highest bootstrap value of 100%. Overall, except for isolate P4, which is identified to be a putative *V. harveyi*, all other isolates belong to different bacterial genera.

Phenotypically, *Vibrio* spp. are motile, Gram-negative bacteria that are curved-rod in shape, facultatively aerobic, catalase-positive, and with a fermentative and respiratory metabolism (Weil and LaRocque, 2020). To isolate Vibrios from the mangrove crabs with loose shell syndrome, TCBS agar was used as a medium as it is widely used to isolate *Vibrio* spp. from environmental samples. Sucrose-fermenting Vibrios, such as *V. cholerae*, form yellow colonies on the medium, whereas the non-sucrose-fermenting Vibrios, including *V. parahaemolyticus*, form green colonies. Among the five bacterial isolates selected for phenotypic characterization, two produced yellow, and three produced green colonies. All isolates were Gram-negative. They also tested positive for catalase and motility. For other biochemical properties, the results can vary depending on the *Vibrio* species. Typically, Vibrios test negative for hydrogen sulfide (H_2S) production due to their inability to reduce thiosulfate to sulfide in SIM medium. In bacteria that can reduce thiosulfate, the resulting sulfide reacts with iron in the medium to form a black precipitate, indicating H_2S production. Unlike Vibrio species, bacteria such as Salmonella and Proteus produce this black precipitate (Paydar, 2013). Only Isolate P4 tested negative for H_2S production, suggesting it could be a putative *Vibrio*, while the others may belong to different bacterial genera. Isolate P4 also displayed the

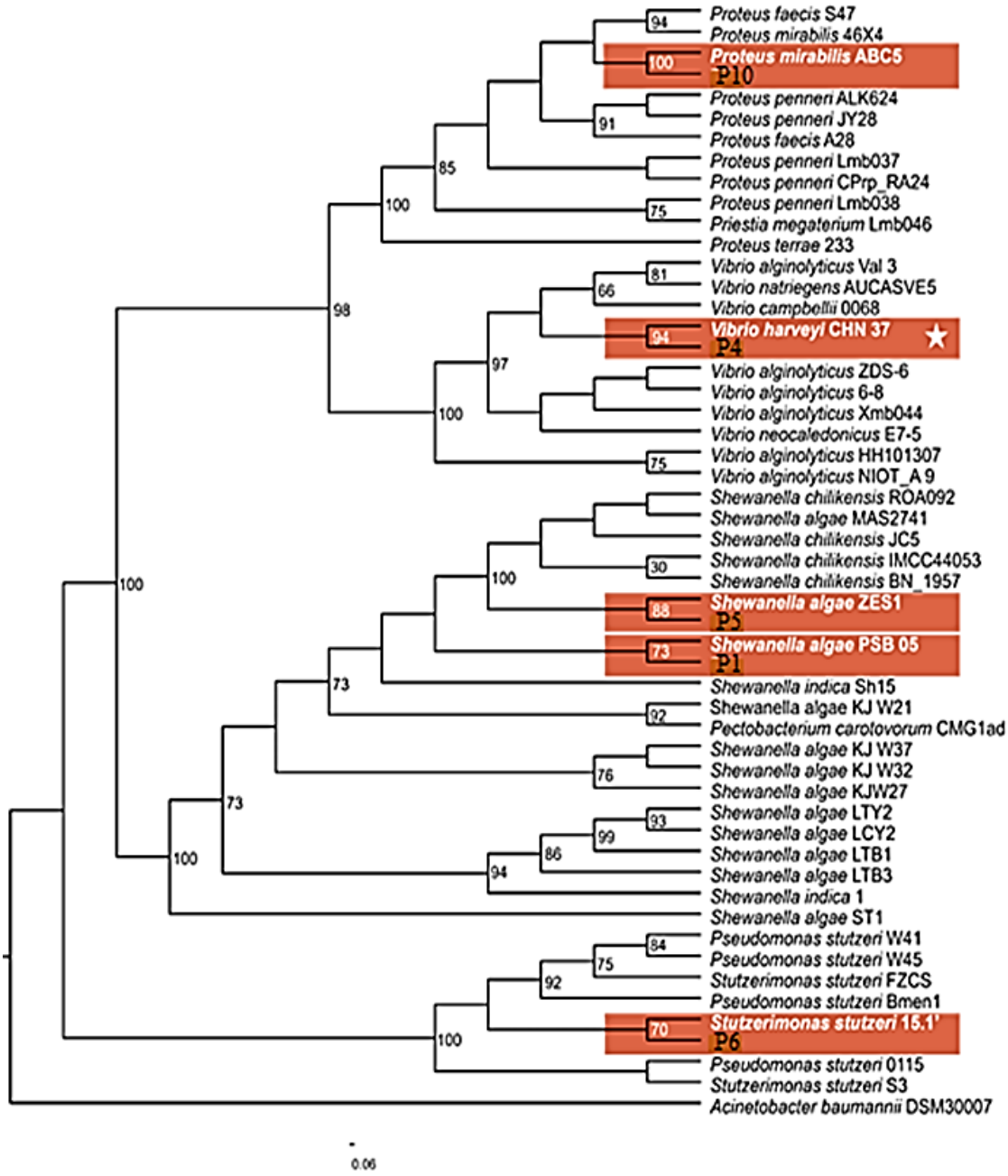

Figure 2. Phylogenetic tree of the 16S rRNA sequences of the selected bacterial isolates.

characteristic comma-shaped morphology under the microscope. These findings question the selectivity of TCBS agar as a culture medium for the isolation of Vibrios.

In a study conducted by Shikongo-Nambabi (2012), it was found that many of the putative *Vibrio* isolates obtained did not belong to the bacterial group. Their findings underscore the need to improve the selectivity of TCBS to inhibit the growth of non-Vibrios such as *Pseudomonas*, *Aeromonas*, *Shewanella,* and members of *Enterobacteriaceae*. Another selective culture medium used in this study for screening Vibrios was the Chromogenic Vibrio Agar (CVA). This selective medium is designed to differentiate three types of Vibrios based on unique enzyme activity. ß-glucosidase activity results in blue-green colonies, typical of *V. parahaemolyticus*. Red or pink colonies indicate ß-galactosidase activity and are observed in *V. cholerae* and *V. vulnificus*. Lastly, the colorless colonies are identified as *V. alginolyticus*,

whose ß- galactosidase expression is inhibited by high sugar concentrations. All the isolates appeared to have characteristics expected of Vibrios, displaying either pink-rose or colorless colonies. However, considering the positive results of most of the isolates in the H_2S production test, only isolate P4 is likely to be *Vibrio*.

To identify the bacterial isolates at the molecular level, their DNA was extracted and subjected to PCR analysis for the detection of the *pirA* gene, which is indicative of a specific strain of *V. parahaemolyticus* known to cause Acute Hepatopancreatic Necrosis Disease (AHPND). It is a devastating disease affecting populations of penaeid shrimp (Soto-Rodriguez et al., 2022). The *pirA* gene encodes a secreted protein, a major virulence factor inducing the disease. The agarose gel electrophoresis results revealed that none of the isolates were positive for the target gene. The primers developed by Castroverde et al. (2006) were chosen for their ability to detect a 320-bp segment of the *vhh* gene in *Vibrio* spp. isolates originating from the Philippines, which have exhibited pathogenicity towards shrimps. The 16s rRNA sequence analysis of the bacterial isolates further confirmed their identities. Isolate P4 was identified as a putative *V. harveyi*, but the remaining isolates were non-Vibrios. Isolate P1 and P5 were closely related to *Shewanella* algae, isolate P6 to *Stutzerimonas stutzeri*, and isolate P10 to *Proteus mirabilis*. These results support the study by Shikongo-Nambabi (2012), reinforcing that TCBS agar is not sufficiently selective for isolating Vibrios. A more selective medium aside from TCBS agar should be employed to isolate pathogenic Vibrios from mangrove crabs with loose shell syndrome. Furthermore, employing a number of phenotypic tests for screening is advisable before proceeding to molecular techniques to confirm their identity accurately.

Conclusion

In conclusion, pathogenic Vibrios, particularly *V. harveyi* harboring the *vhh* gene, can be isolated from mangrove crabs exhibiting loose shell syndrome. The findings in this study provide preliminary evidence for further investigations to delineate the pathogenic mechanisms of Vibrios and assess how they contribute to the underlying symptoms of the disease, whose cause remains elusive. Future studies should focus on performing experiments using these Vibrios to ascertain whether these bacteria are the etiologic agents of this condition in mangrove crabs.

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