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Investigation of Bacterial Infections in Farmed Mud Crab (*Scylla paramamosain*) in Ca Mau Province, Viet Nam

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ABSTRACT

This study aimed to identify the bacterial pathogens associated with abnormal symptoms in farmed mud crabs (Scylla paramamosain) in Ca Mau province. There were 161 isolates of bacteria obtained from blood, hepatopancreas and muscle of 202 collected mud crabs using TCBS and TSA medium supplemented 1.5% NaCl. A total of 11 representative isolates based on morphological features were identified using 16S rRNA sequencing. There were six different species including Vibrio parahaemolyticus, Vibrio alginolyticus, Vibrio harveyi, Photobacterium damselae, Shewanella algae and Staphylococcus saprophyticus were identified, in which Vibrio spp. were the most dominant (63,63%). These bacterial species, except S. algae, have been documented as causative agents of bacterial diseases in mud crabs. Furthermore, the phylogenetic analysis indicated that Vibrio species isolated in the present study were closely related to each other as well as the minimal difference between isolates and other selected reference strains.

1. INTRODUCTION

Mud crab (*Scylla paramamosain)* is broadly distributed in the tropical Indo-Pacific area and commonly found in mangrove forests and estuaries. Recently, the culture of mud crab has increased in interest in many countries due to its high economic value and market demand (Shelley & Lovatelli, 2011; Waiho et al., 2018). However, mud crab farming has been suffered excessive economic loss due to disease outbreak (Hungria et al., 2017; Jithendran et al., 2010; Saha et al., 2023; Xie et al., 2021c). Parasites, bacteria and viruses have been reported as emerging pathogens causing high mortality rate to the mud crab aquaculture practices (Coates & Rowley, 2022). Notably, bacterial infections were recorded as the most prevalent in crab culture (Rogers et al., 2015).

Mud crabs (*S. paramamosain*) are mostly cultured in coastal provinces of Vietnam such as Ben Tre, Tra Vinh, Soc Trang, Bac Lieu, Kien Giang, and Ca Mau (Ca Mau Provincial Department of Agriculture and Rural Development, 2024). Particularly, Ca Mau province is considered the "capital" of mud crabs for its biggest cultured areas, highest production and best quality (Ministry of Agriculture and Rural Development [MARD], 2020). Since 2020, mud crabs have been reported abnormally dead, reaching a mortality rate of 90% in Nam Can and Ngoc Hien districts, where are the main crab culture areas (MARD, 2024). In 2022, the massive death ranged from 30 to 100%, resulting in

damaging 90% of the total production of mud crab in Dam Doi and Nam Can districts (MARD, 2024). According to Ca Mau Provincial Department of Fisheries (2023), the disease has occurred in cycles or episodic events, frequently in the dry season. In early 2024, the massive death of farmed mud crabs farm recurred in Ca Mau, seriously in Nam Can and Dam Doi district has been reported to reoccur with a damaged area of 600 ha (over 50% of the total mud crab culture area) (MARD, 2024).

Due to the servere deadth of the cultured mud crabs, various pathogenic potentials have to be assessed to be more understand for further decision-making in disease management. In this paper, we focus on bacteria associated with clinical symptoms of infectious crabs collected in Ca Mau were investigated.

2. MATERIALS AND METHOD

2.1. Sample sites

Mud crab collections were performed in 5 districts of Ca Mau province including Ngoc Hien, Nam Can, Cai Nuoc, Dam Doi and Thai Binh of Ca Mau province. These sampling sites were selected based on the locations of mass mortality reported in some recent last crops and the availability of diseased mud crabs.

2.2. Sample collection

The mud crabs (*S. paramamosain)* with abnormalities as lethargy, loss of appetite, discoloration of carapace, soft-shell and tremor signs in walking legs were collected from March 2023 to March 2024.

2.3. Detection of bacterial infection

2.3.1. Bacterial isolation

The mud crabs were aseptically analyzed within 2-4 hours after being collected. Bacteria were isolated from haemolymph, hepatopancreas and muscle. Briefly, the outer surface of the crab was disinfected by spraying 70% ethanol, and then haemolymph was withdrawn from the fourth or fifth walking leg. A drop of hemolymph was plated on Tryptone Soya Agar (Himedia) supplemented with 1.5% NaCl (TSA^+) plate and a Thiosulfate-Citrate-Bile Salt Sucrose Agar (Himedia) plate, respectively. The muscle and hepatopancreas were aseptically removed and swabbed on TSA^+ and TCBS plates. Plates were incubated for 24-48 hrs at 28°C. Based on the morphological feature, colonies were randomly picked and purified by re-streaking method. The pure isolates were stored in glycerol stock at -80°C.

2.3.2. Bacterial identification using 16s rRNA gene sequencing

DNA was bacterial isolates were extracted using boiling method. Brieftly, single colony was picked and placed into an eppendorf containing 100 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), then incubated at 100°C for 10 min. The mixture were snap cooled on ice for 5 min and centrifuged 13000 rpm for 5 min. The upper aqueous amulsions was changed to a new eppendorf and served as DNA template for PCR amplification.

Two universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') were used to amplify the target 16S rRNA gene in bacteria . The PCR reaction (50 µL) consisted of 1X Taq buffer; 1,5 mM $MgCl_2$; 200 µM dNTPs; 1,5 U *Taq* DNA polymerase (Promega); 0,4 µM each primer and 1 µL DNA template. The PCR mixture was incubated at 95°C for 5 min and then for 30 cycles (94°C for 1 min, 56˚C for 1 min, and 72°C for 1 min 45 sec), followed by a final 10 min extension at 72°C using a thermal cycler (Biorad, USA). The PCR products were analyzed on 1% agarose gel to verify the presence of amplicons at expected size. The PCR products subsequently purified and sequenced by First Base Pte Ltd. (Malaysia). Sequences from forward and reverse primers were analyzed using FinchTV software and compared for homology using nucleotide BLAST on NCBI database (https://www.ncbi.nlm.nih.gov/). A phylogenetic (neighbour-joining) tree was constructed using MEGA X software following multiple alignments of the 16S rRNA sequences in this study and their related species available on Genbank.

3. RESULTS AND DISCUSSION

3.1. General information of mud crab samples

A total of 202 mud crabs exhibited gross signs as soft-shell, leg trembling, discoloration on carapace, muscle wasting, pale hepatopancreas and endoparasite presenting in organs (Fig. 1). The crabs were sampled during dry season (from March to May each year). The crab size ranged from 73.14 to 104 mm in carapace width and 76.81 to 253 g in weight. All mud crab samples were collected at the the improved extensive model.

3.2. Bacterial isolation

Of the 202 samples analyzed, the bacterial infections were recorded at a high rate (>90%). On TSA^+ plates, the infection rate was 96,37%, 97,59% and 100% in haemolymph, hepatopancreas and muscle, respectively, while the presence of *Vibrio* spp in haemolymph, hepatopancreas and muscle on TCBS plates was recorded as 30.12%, 64.47% and 81.93%, respectively. Based on colony morphology, 161 representative colonies were picked for Gram staining and basic biochemical parameters as motility, oxidase, and catalase tests. Most of the isolated bacteria were rod-shaped, Gram negative (93.79%), the rest were cocci-shaped and Gram positive (6.21%). In previous studies, Gram-negative bacteria were frequently reported to be the dominant bacteria infected in mud crab ponds (Jithendran et al., 2010). All of the bacterial isolates (161/161) were positive on the oxidase test, 97.52% (157/161) and 59.01% (95/161) isolates were positive on the catalase and motility test respectively (Fig. 2). A final of 11 representative bacterial isolates (2 isolates from Dam Doi, two isolates from Ngoc Hien, two isolates from Nam Can, three isolates from Thoi Binh and two isolates from Cai Nuoc district) were selected for bacterial identification using 16S rRNA gene sequencing.

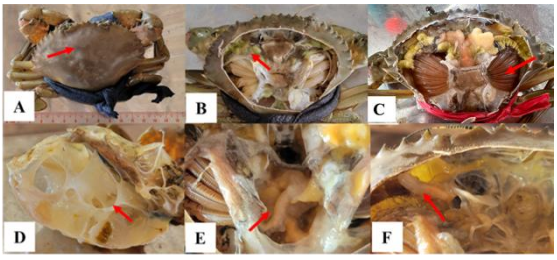

Figure 1. Gross signs of mud crab samples (A) Red carapace, (B) Pale hepatopancreas, (C) Black gills, (D) Muscle necrosis, (E) and (F) Endoparasites in organs

3.3. Diversity of isolated bacteria associated with infected mud crab

Amplification of 16S rRNA gene yielded approximately 1.500 bp amplicon from the extracted DNA. After DNA analysis, the results from BLAST-N search showed that the DNA sequences of bacteria isolated from infected mud crab showed high similarity value, from 98.54% to 100% identity. The only homology level 98.54% was appointed to DD24 2-1 isolate which similar to *Staphylococcus saprophyticus*. The remaining isolates exhibited 100% homology to the reference bacteria in database as shown in Table 1. Among 11 bacterial isolates, *Vibrio* spp. have been identified in 7 (63.63%) of them and distributed in all sample locations. Specifically, 3 *V. parahaemolyticus* isolates were identified (Cai Nuoc: 1 and Nam Can: 2); 3 *V. alginolyticus* (each distributed in Dam Doi, Ngoc Hien and Thoi Binh) and the other one identified *V. harveyi* in Ngoc Hien. The four remaining isolates were recognized as 2 *Photobacterium damselae* (each distributed in Cai Nuoc and Thoi Binh), 1 *Shewanella algae* in Thoi Binh and 1 *Staphylococcus saprophyticus* in Dam Doi. Regards to gross observation, our identified bacteria which associated with infected mud crabs were in line with previous studies, that reported the clinical signs of those bacterial infection in mud crab as lethargy, soft carapace, decoloured of carapace or legs and spots on the surface (Jithendran et al., 2010; Saha et al., 2023; Souza Valente & Wan, 2021; Xie et al., 2021c, 2021a). However, these results revealed that the identified bacteria associated with diseased mud crabs in Ca Mau province were less diverse than that found in cultured and wild crabs in Malaysia (9 bacterial species) (Najiah et al., 2010), India (Jithendran et al., 2010) and Indonesia (5 species) (Sarjito et al., 2022).

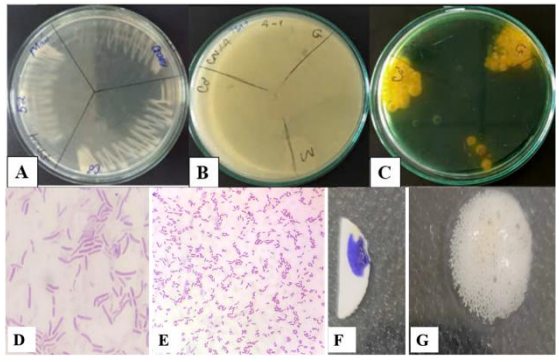

Figure 2. Morphology of the isolated bacterial colonies, cells and biochemical test (A) and (B) Bacterial colonies on TSA^+ plates, (C) Bacterial isolates on TCBS plate, (D) and (E) Gram negative, rod-shape, (E) Oxidase test, (F) Catalase test

Table 1. Molecular characterization of 8 representative bacterial isolates associated with infected mud crabs

No.	District	Isolate code	Gross signs	Morphology features		Biochemical characteristics			Bacterial identification	Identity (%)	Query cover (%)	Acc. Number	Query length (bp)
				Colony	Cell	M	C	O					
1	Cai Nuoc	CN24 2-1	Lethargy, soft-shell, undefined white chain in abdomen	White, sliding	Rod/ Gram (-)	+	+	+	*Vibrio parahaemolyticus*	100	100	MK102631.1	1364
2		CN24 2-2	Lethargy, darken gills	White, round	Rod/ Gram (-)	+	+	+	*Photobacterium damselae*	100	100	MT075581.1	1367
3	Dam Doi	DD24 2-1	Soft-shell,	Cream, round	Cocci/ Gram (+)	-	+	+	*Staphylococcus saprophyticus*	98,54	100	MN077137.1	1348
4		DD24 6-1	Decolored carapace, muscle necrosis, pale hepatopancreas	White, sliding	Rod/ Gram (-)	+	+	+	*Vibrio alginolyticus*	100	100	MN938185.1	1364
5	Nam Can	NC24 2-2	Decolored carapace, soft-shell	White, sliding	Rod/ Gram (-)	+	+	+	*Vibrio parahaemolyticus*	100	100	CP051113.1	1335
6		NC24 6-1	Decolored carapace and cheliped, pale hepatopancreas	White, sliding	Rod/ Gram (-)	+	+	+	*Vibrio parahaemolyticus*	100	100	KT023517.1	1312
7	Ngoc Hien	TAT24 2-3	Trembling in legs	White, sliding	Rod/ Gram (-)	+	+	+	*Vibrio alginolyticus*	100	100	MT299658.1	1366
8		TAT24 3-5	Lethargy, pink coloration in tissue	White, round	Rod/ Gram (-)	+	+	+	*Vibrio harveyi*	100	100	MG593678.1	1363
9	Thoi Binh	TB24 5-1	Lethargy, pink coloration in tissue	Creamy, large round	Rod/ Gram (-)	+	+	+	*Shewanella algae*	100	100	MH368435.1	1358
10		TB24 5-2	Lethargy, tissue necrosis	White, sliding	Rod/ Gram (-)	+	+	+	*Vibrio alginolyticus*	100	100	PP779835.1	1326
11		TL 2-1	Lethargy	White, round	Rod/ Gram (-)	+	+	+	*Photobacterium damselae*	100	100	ON564500.1	1418

Three genuses of Vibrionaceae including *Vibrio, Photobacterium* and *Shawanella* were frequently found in mud crabs (Jithendran et al., 2010; Souza Valente & Wan, 2021). Three species of *Vibrio*, namely *V, alginolyticus, V. parahaemolyticus*, and *V. harveyi* were detected in this study and these bacteria have been reported as probable pathogens causing shell-disease syndrome in mud crab (Rowley & Coates, 2023; Sindermann, 1989). *Vibrio alginolyticus* MF680287. It was reported as the causative agent of shell disease in mud crab *Scylla serrata* grown pond in India (Gunasekaran et al., 2019). Recently, Kwok et al., (2024) have demonstrated *V. alginolyticus* as the main infectious agent leading to high mortality (up to 75%) in both experimental challenges and farm practice in Hongkong. In addition, other crabs like swimming crab (Shi et al., 2019), gazami crab (*Portunus trituberculatus)* (Xia et al., 2018) were also reported as susceptible hosts of *V. alginolyticus*. Relating to vibriosis in mud crab, *V. harveyi* has been noticed as a causative agent in mud crab farmed in Central Java, Indonesia (Sarjito et al., 2022) and West Bengel, India (Saha et al., 2023). Seriously, *V.*

harveyi could cause 90-100% mortality in zoe stage of swimming crabs (Zhang et al., 2014). *Vibrio parahaemolyticus*, the last identified *Vibrio* is this study, was frequently found as a main pathogen with high virulence in mud crab *S. paramamosain* cultured in China (Xie et al., 2021d), in Bangladesh (Aftabuddin et al., 2013) and in Thailand (Srimeetian et al., 2017). In addition, *V. parahaemolyticus* was also detected in both blue crab (*Callinectes sapidus*) and in the seawater samples collected in Maryland Bay, USA (Smalls et al., 2023).

Photobacterium damselae, a pathogenic bacterium infected to various aquatic animals and humans (Rivas et al., 2013) was also found in the present study. In previous studies, *P. damselae* was found to be one of the most virulent pathogens in fish and shrimp (Liu et al., 2016; Terceti et al., 2016; Vaseeharan et al., 2007). Till 2017, *P. damselae* was first reported from diseased cultured mud crabs in Zhejiang, China (Xie et al., 2021b). Through bacterial challenge experiments, *P. damselae* was confirmed to be virulent for mud crabs.

Shewanella algae is considered an emerging human pathogen (Fernandes et al., 2023; Holt et al., 2005). *S. algae* was reported as a causative pathogen in black spot disease in white leg shrimp *Penaeus vannamei* (Cao et al., 2018). In addition, *S. algae* has affected various fish species in India (Pathinathan, 2024). Although *S. algae* has not been reported as a pathogenic bacterium in crab, the increasing prevalence of this bacterium found in shrimp seafood (Dutta et al., 2020) and in blue crab *C. sapidus* product should be paid attention (Rubini et al., 2024).

Staphylococcus saprophyticus, the only Gram-positive bacteria was detected in this study. *S. saprophyticus* has been described as an opportunistic pathogen that causes urinary tract infections in human (Dutta et al., 2020). A number of studies have reported this bacterium in aquatic environment as polluted residues (Basso et al., 2014; Harakeh et al., 2006). However, *S. saprophyticus* was found as causative agent causing lesions in multiple organs of hybrid sturgeons in China (Wu et al., 2023). More important, the co-infection of *S. saprophyticus* and reovirus in fattenting mud crab (*Scylla serrata*) cultured along the southern East coast of India has recently been reported (John et al., 2024).

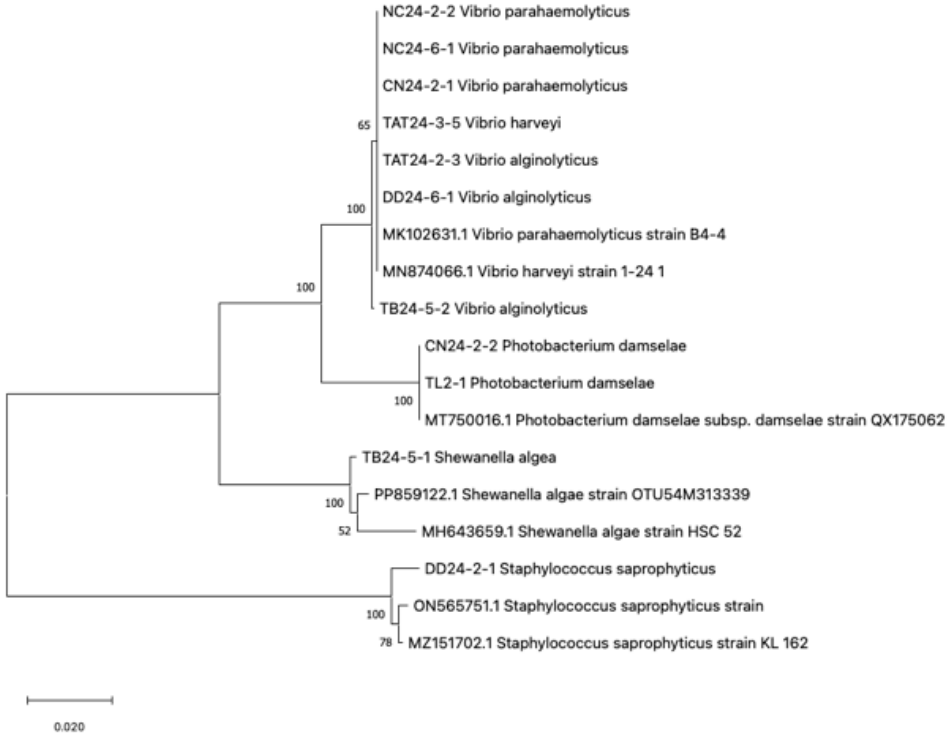

Figure 3. Phylogenetic of the bacteria associated with bacterial diseases in mud crabs in Ca Mau

The phylogentic analysis of 18 nucleotide sequences (11 from this study and 7 obtained from NCBI Genbank) to present the relationship among our isolates. According to the branching patterns, all of isolates are clearly divided into 2 major clades in which clade 1 consisting of all *Vibrio* species, *Photobacterium* species and *Shewanella* species while clade 2 is only *Staphylococcus saprophyticus* (Fig. 3). The analysis revealed that *Vibrio* species in the present study were relatively concentrated, except for *V. alginolyticus* TB24 5-2 isolated in Thoi Binh. In addition, this clade also displayed extremely short tree branches with respect to reference taxa, indicating minimal difference among the analyzed sequences and others originating from either China or India. Notably, our *P. damselae* sequences showed showed distinct genetic relationship to *P. damselae* with accession number MT750016.1 originating from China and *S. saprophyticus* DD24-2-1 isolate is more similar to *S. saprophytycus* (acc. nr ON56575.1) identified as infectious pathogen on mud crab in India.

According to the above results, more information about environmental conditions which lead to the disease outbreak, as well as the correlation of bacterial pathogens with other microbial pathogens could help in the prevention and management diseases in mud crab farm.

4. CONCLUSION

In conclusion, there were 11 bacteria found associated with bacterial diseases in mud crabs in Ca Mau province in the present study. The results showed a close relationship to *V. parahaemolyticus*, *V. alginolyticus*, *V. harveyi*, *S. algae*, *P. damselae* and *S. saprophyticus* with high homology. These bacteria are well-known as virulent bacterial pathogens to mud crabs, with the exception of *S. algae*.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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