Antibiotic resistance and molecular identification of dominant bacteria associated with loose shell syndrome in mangrove crabs (*Scylla* spp.)

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Abstract. The emergence of loose shell syndrome (LSS) in Philippine mangrove crabs poses a serious threat to both the aquaculture industry and the delicate ecosystems it relies on. This study aimed to identify dominant bacteria associated with LSS and assess their antibiotic resistance to guide treatment and prevention strategies while safeguarding public health. Mangrove crab samples with loose carapace were collected from the province of Capiz, Philippines. Dominant bacteria that grew on Thiosulfate–Citrate–Bile Salts–Sucrose (TCBS) and modified chitin agar were isolated and identified by sequencing of the16S rRNA. Antibiotic susceptibility of the dominant bacterial isolates was performed using the agar disk diffusion method. Dominant bacteria that grew on TCBS agar included putative *Vibrio*, *Proteus*, *Shewanella*, and *Stutzerimonas*. The isolates had antibiotic resistance indices (ARI) ranging 0.8 to 1.2. Chitinolytic bacteria were dominated by *Vibrio*, *Shewanella*, and *Brevundimonas*. Both *Vibrio* and *Brevundimonas* exhibited resistance to gentamicin, ciprofloxacin, and norfloxacin, while *Shewanella* showed resistance to ciprofloxacin. The presence of multi-drug resistant bacteria in LSS-affected crabs highlights the urgent need for effective health management strategies in the industry while mitigating the risks associated with antibiotic resistance to protect both the sustainability of mangrove crab aquaculture and the health of consumers.

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1 Introduction

Shell disease has been documented in a number of economically significant crustaceans, including mangrove crabs, *Scylla* spp. [1]. This disease is also associated with a variety of environmental circumstances [2]. The development of shell disease is believed to be caused by multiple influencing factors, predominantly linked to physical damage to the epicuticular layer and chitinolytic processes from colonizing bacteria [3-6], and fungi [7]. Additionally, external elements like heightened nutrient concentrations, water and soil contaminants, and diminished oxygen levels, play contributory roles [1, 8-9]. While shell disease is typically superficial and hence, lack access to the underlying tissues, evidence suggests that severe cases may lead to mortality through the infiltration of bacteria into the hemolymph of the crab due to the compromised integrity of the protective cuticle.

Mangrove crab cultivation in brackishwater ponds in the Philippines has experienced a decline in production following the emergence of a novel variant of shell disease termed by the study as "loose shell syndrome [10]. Local mangrove crab pond operators detail distinctive symptoms atypical among known shell disease types namely: a soft and loose carapace, muscle atrophy, and an overall reduced body mass.

Despite the evident harm of loose shell syndrome on the local aquaculture industry and the mangrove crab population, its etiology is yet to be elucidated in the scientific literature. Therefore, this study aimed to improve our understanding of the underlying causes of loose shell syndrome in mangrove crab by identifying putative Vibrios and chitinolytic bacteria that are associated with this condition.

2 Materials and methods

2.1 Sampling and bacterial isolation

Five mangrove crabs showing signs of shell disease were collected from Roxas City, Capiz, Philippines and transported to the University of the Philippines Visayas Microbiological Laboratory. In the laboratory, gill tissues were extracted from the crabs under sterile conditions, weighed, and processed for analysis. The samples were homogenized in a saline solution, serially diluted, and plated onto specialized agar media: Thiosulfate–Citrate–Bile Salts–Sucrose agar for isolating putative Vibrios, and modified chitin agar for isolating bacteria with chitinolytic activity. After incubation at 27°C for 24 hours, distinct colonies were selected from the agar plates at the highest dilution factor – yellow and green colonies from TCBS agar, and colonies with clear zones from modified chitin agar [11]. These selected colonies were then re-streaked to Nutrient agar (NA) plates containing 1% sodium chloride (NaCl) to obtain pure isolates and stored at 4°C for further assays.

2.2 Antibiotic susceptibility

The agar disc diffusion assay [12] was used to test the susceptibility of the dominant bacterial isolates to commonly used antibiotics in aquaculture. The antibiotics were the

following: chloramphenicol (C), 30 µg; ciprofloxacin (CIP) 5 µg; gentamicin (CN), 10 µg; norfloxacin (NOR), 10 µg; tetracycline (TE), 30 µg.

The bacterial isolates were inoculated into 10 ml nutrient broth (NB) with 1% NaCl and incubated at 28°C for 24 h. Following this, the density of every bacterial suspension was adjusted to roughly 1.5 x 10^8 CFU/ml through the addition of sterile NSS until the turbidity matched that of a 0.5 McFarland standard solution. A sterile cotton swab was dipped into the bacterial suspension and streaked evenly onto the surface of NA-1% NaCl. The inoculated plates were left undisturbed on a flat surface for 1 h to allow adherence of the bacteria [13].

The antibiotic discs were strategically positioned on the plates using sterile forceps, and the agar plates were incubated at 28°C for 24 h. After the incubation period, zones of inhibition were assessed through the measurement of clear zones and compared to the zone of inhibition interpretative standards provided by the Clinical and Laboratory Standards Institute (CLSI) [14] (2015). Resistance of the bacterial isolates to the antibiotics was then quantified in terms of a modified antibiotic resistance index (ARI), which is calculated as the average score assigned based on the susceptibility of a bacterial isolate to each antibiotic tested. Scores are assigned as 0 for susceptible, 1 for intermediately susceptible, and 2 for resistant. The ARI is obtained by dividing the sum of these scores by the total number of antibiotics tested, providing a value between 0 and 2. This index reflects the average resistance level of a bacterial isolate, with higher values indicating greater resistance. All assays were done in triplicate.

2.3 Molecular identification of bacteria

Genomic DNA was isolated from an overnight bacterial culture in 1.5 ml microfuge tube using a commercial DNA extraction kit (Invitrogen™ PureLink™ Genomic DNA Mini Kit). The genomic DNA samples were kept at -20°C for subsequent PCR amplification and sequencing.

To amplify the 16S rRNA, primers (Forward: GAGAGTTTGATCCTGGCTCAG; Reverse: CTACGGCTACCTTGTTACGA) [15] were used in a 25 uL polymerase chain reaction (PCR) mix. The PCR reaction was comprised of 2 uL (10-15 ng) DNA as template, 2 uL of each primer (5 pmol), 2.5 uL PCR buffer, 1.5 uL 2mm dNTP, 1 uL 50mm MgCl2 and distilled water. PCR amplification was performed using an initial denaturation of 95°C for 3 min, followed by 35 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 55°C, and elongation for 1 min at 72°C, and a final elongation step at 72°C for 5 min completed the reaction. The PCR products were cleaned and submitted for sequencing to Macrogen (Korea). Each isolate was putatively identified using BLASTn search and were compared with other 16S rRNA sequences of related strains from NCBI Genbank (blast.ncbi.nlm.nih.gov) database.

3 Results and Discussion

Five colonies that grew on TCBS agar plates at the 10^{-5} dilution were obtained (Fig. 1). Two colonies are sucrose fermenters (isolates P2 and P4), while three colonies are non-sucrose fermenters (isolates P1, P3 and P5).

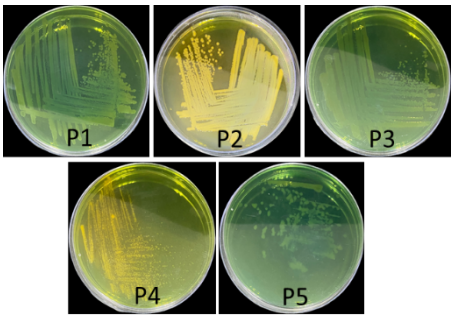

Fig. 1. Dominant bacterial colonies in TCBS agar.

Chitinase-producing bacteria were also isolated from loose shell crabs as shown in Figure 2. These bacterial isolates formed clear zones or halo in modified chitin agar after staining with 0.1% Congo Red. These isolates exhibited the highest chitinolytic indices among the bacteria that were screened.

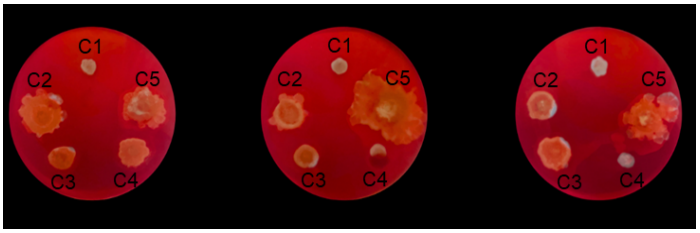

Fig.2. Chitinolytic bacteria from mangrove crabs with loose shell syndrome. Each agar represents replicate sample.

Resistance of the bacterial isolates to the different antibiotics that were tested is shown in Table 1. All the putative Vibrios (P1-P5) are resistant to gentamicin. Only one isolate, P5 is resistant to both gentamicin and ciprofloxacin. The five dominant chitinolytic bacteria (C1 – C5) that were isolated from mangrove crab with loose shell syndrome are resistant to ciprofloxacin. Four of the isolates are resistant to gentamicin, ciprofloxacin and norfloxacin.

Table 1. Antibiotic resistance of the bacterial isolates from mangrove crabs with loose shell syndrome

Isolate	Resistance to Antibiotics	**ARI**
P1	Gentamicin	0.8
P2	Gentamicin	0.8
P3	Gentamicin	0.8
P4	Gentamicin	0.8
P5	Gentamicin, Ciprofloxacin	1.2
C1	Gentamicin, Ciprofloxacin, Norfloxacin	1.2
C2	Gentamicin, Ciprofloxacin, Norfloxacin	1.4
C3	Ciprofloxacin	0.4
C4	Gentamicin, Ciprofloxacin, Norfloxacin	1.4
C5	Gentamicin, Ciprofloxacin, Norfloxacin	1.4

The ARI was computed from three independent replicates.

Table 2 shows the molecular identity of the dominant bacterial isolates from mangrove crab with loose shell syndrome. Sequencing of the 16S rRNA showed that only 1 isolate obtained from TCBS agar had high similarity with *Vibrio harveyi*. The other four isolates are putative *Shewanella algae* (2 isolates), *Stutzerimonas stutzeri* and *Proteus mirabilis*. The dominant chitinolytic bacteria that are associated with loose shell syndrome in mangrove crab are *Vibrio*, *Shewanella* and *Brevundimonas*.

Table 2. Molecular identification of dominant bacteria from mangrove crabs with loose shell syndrome

Isolate	Closest species	Identity (%)
P1	*Shewanella algae* PSB 05	73.0
P2	*Vibrio harveyi* CHN 37	94.0
P3	*Shewanella algae* ZES1	88.0
P4	*Stutzerimonas stutzeri* 15.1‘	70.0
P5	*Proteus mirabilis* ABC5	100.0
C1	*Vibrio alginolyticus* strain CIFRI V-TSB1	96.0
C2	*Vibrio natriegens* strain AUCASVE5	97.0
C3	*Shewanella* sp.	97.0
C4	*Brevundimonas* sp. N5(2011)	98.0
C5	*Brevundimonas diminuta*	95.0

In an earlier study of Shikongo-Nambabi [16], it was revealed that many of the putative *Vibrio* isolates obtained did not belong to the bacterial group. This was also evident in the present study. The findings underscore the need to improve the selectivity of TCBS in order to inhibit the growth of non-vibrios, such as *Pseudomonas*, *Aeromonas*, *Shewanella* and members of the Enterobacteriaceae. The identification of dominant chitinolytic bacteria, *e.g.*, *Vibrio*, *Shewanella* and *Brevundimonas*, that are associated with loose shell syndrome in mangrove crab supports the findings of previous studies that these bacterial species possess chitinase [17-19] and are also implicated in some shell diseases in crustaceans [20-22]. Specifically, shell disease syndrome is primarily attributed to the dominance of chitinolytic bacteria, particularly *Vibrio* spp., which act as opportunistic pathogens colonizing the shells of marine crustaceans [23].

The mechanism of the pathogenicity of *Shewanella* is not yet fully elucidated; however, there are certain strains of this species that are capable of generating a range of enzymes such as chitinase, protease, lipase, alkylsulfatase, and elastase [24]. *Shewanella* is also associated with some shell diseases in crustaceans [25-27].

In terms of the sensitivity of the bacterial isolates to the various antibiotics tested, studies have demonstrated that Vibrios are predominantly susceptible to chloramphenicol, ciprofloxacin, and norfloxacin. These bacteria often exhibit intermediate susceptibility or resistance to gentamicin and generally show susceptibility or intermediate susceptibility to tetracycline. The findings of this study are in agreement with studies done previously [28-29]. There were limitations on the number of antibiotics used in the present study; thus, it is recommended that increasing the number of antibiotics for the drug resistance assays should be the point of focus in future studies.

4 Conclusions

Taken together, this study identified the predominance of specific bacteria, including *Vibrio*, *Shewanella*, and *Brevundimonas* species, in mangrove crabs exhibiting loose shell syndrome. The chitinolytic activity observed in these bacterial isolates suggests a potential mechanism for shell degradation and weakening in affected crabs. This necessitates further investigations using Koch's postulates to elucidate the pathogenic mechanisms of these bacteria and assess how they contribute to the underlying symptoms of the disease. Additionally, the study highlights the presence of antibiotic-resistant bacteria, emphasizing the need for future research focused on developing effective disease prevention and treatment strategies to ensure seafood safety and protect public health.

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