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## Shell disease in the Freshwater crab, *Barytelphusa cunicularis*

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### ABSTRACT

The current study deals with the isolation and identification of bacterial and fungal pathogens, from lesioned carapace and limb of the freshwater crab *Barytelphusa cunicularis*, inhabiting the paddy fields of Mananthavady, Wayanad, Kerala. The bacterial flora was isolated by pour plate method and phenotypic identification was made based on morphological features and biochemical tests. The fungal pathogen was isolated from Sabouraud dextrose agar medium and morphologically identified using Lactophenol cotton blue stain. From the infested carapace, bacteria belonging to nine genera - *Aeromonas*, *Alcaligenes*, *Bacillus*, *Chromobacterium*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Micrococcus* and *Pseudomonas* and one fungal pathogen *Aspergillus* sp. were identified. Infested limb showed the presence of *Pseudomonas* and *Escherichia coli*. The present study on the isolation and identification of microbes from infested carapace and limb will aid in understanding the pathogen profile and its prevalence in wild populations, so as to determine its suitability for human consumption.

**Keywords:** *Barytelphusa cunicularis*, Chitinolytic bacteria, Pour plate method, Shell disease.

**Abbreviations:** H<sub>2</sub>S - hydrogen sulphide gas, MR - methyl red test, NA - nutrient agar medium, SDA - Sabouraud dextrose agar medium, SEM - scanning electron microscopy, TSI - triple sugar iron test, THB - total heterotrophic bacterial load, VP - Voges Proskauer test.

### 1. Introduction

Shell disease is a progressive degradation of the crustacean cuticle characterized by external melanized focal lesions of varying size and severity. Exoskeletal erosions have been reported as: shell disease syndrome, black necrotic disease, box burnt disease, black mat syndrome, rust disease, brown spot, black spot, burn spot, tail rot and spot disease. It can occur in nearly all freshwater and marine crustaceans, usually at low frequency, except when host animals are stressed by poor environmental conditions caused by intensive aquaculture, animal impoundment or water polluted by chemicals, sewage or heavy metals [1, 2, 3]. Investigations have indicated that sand abrasion injuries, predation or cannibalism act as a catalyst for shell disease [4, 5, 6, 7]. Shell infestation by biological agents such as bacteria [8, 9], fungi [10], dinoflagellates [11] and foulers [12] is a major problem in aquaculture, causing heavy economic losses.

Shell diseases of aquaculturally important marine decapods are extensively studied, owing to their commercial importance [13, 14, 15, 16]. A great majority of cases have been linked to common Gram-negative genera of chitinolytic bacteria such as *Achromobacter*, *Acinetobacter*, *Aeromonas*, *Plesiomonas*, *Pseudomonas* and *Vibrio* [4, 17]. Luminous chitin-digesting bacteria such as *Photobacterium* sp., *Moraxella* spp. and *Vibrio anguillarum* caused exoskeletal lesions in the tanner crab, *Chionoecetes tanneri* [18]. Histological, histopathological and scanning electron microscopic observations revealed that necrotic lesions were caused by Gram-positive bacilli in *C. opilio* [19]. Studies have indicated that fungi are occasionally involved in the lesions [20, 21]. Alderman [22] has demonstrated the presence of *Fusarium solani* in the exoskeletal lesions of cultured lobsters. Black mat syndrome caused by the ascomycete fungus *Trichomaritis invadens* was identified in *C. bairdi* [23, 24]. In the blue crab, *Callinectes sapidus*, shell lesions reach the membranous layer of the exoskeleton, coalesce with other lesions and allow secondary infection to invade soft tissues [25]. In severe cases of shell infestation, the animals show signs of

weakness, anorexia, loss of limbs, lower fecundity and mortality [26, 27]. In crustaceans, instances of exoskeletal chitin degradation were reported in less than 10% of natural populations [21, 28, 29].

Shell disease of freshwater crustaceans, crabs in particular, is a comparatively less researched area. There are few reports on the shell diseases of economically important freshwater prawns and crayfishes [30, 31, 32, 33]. Scanning electron microscopic observations of the exoskeletal black spots in cultured penaeid shrimps were described by Yang *et al.* [34]. From the Indian subcontinent, shell disease studies are restricted to prawns and shrimps of aquaculture importance. Abraham and Manley [35] and Abraham *et al.* [36] investigated the exoskeletal lesions in cultured *Penaeus indicus*. Shell disease and tail necrosis in *Macrobrachium rosenbergii* and *P. monodon* from culture ponds of coastal Andhra Pradesh were studied by Jayasree *et al.* [37, 38]. Tail rot disease was reported in *M. idella idella* from Vellar estuary in Tamil Nadu [39].

The freshwater crab, *Barytelphusa cunicularis* (Westwood 1836), inhabiting stony crevices adjoining paddy fields, streams and rivulets, is a readily available source of animal protein to the local tribal population. It is important to identify and characterize the microbes responsible for shell diseases in freshwater crabs, not only because they are edible, but also for their irreplaceable role in the ecosystem services.

## 2. Materials and Methods

### 2.1 Animals

Adult intermoult crabs (CW 7.5 cm-8.5 cm) (February-March 2011) were collected from the stony crevices of catchment areas adjacent to paddy fields of Mananthavady, Wayanad, Kerala. Water and sediment samples were collected from the same habitat in sterile polythene bags. The uninfested and infested crabs were kept separately in tubs and acclimatized to laboratory conditions for two to three days. They were fed *ad libitum* with beef liver and cooked egg white. The infested portions around the carapace and limb were carefully dissected out using sterile scissors and forceps, homogenized and serially diluted. Serial dilutions from carapace and limb of uninfested crabs, water and sediment samples were also prepared.

### 2.2 Isolation, Identification and Enumeration of total heterotrophic bacterial (THB) load

One ml each of the serially diluted sample (infested and uninfested carapace and limb, water and sediment) was poured into separate nutrient agar (NA) plates to isolate bacteria by pour plate method. The samples were incubated at 37 °C for 24 h. After 24 h, morphologically distinct bacterial colonies were counted (THB load). Pure cultures from randomly isolated colonies were transferred to NA slants and stored at 4 °C for further use.

The isolated bacteria were identified based on morphological features (observed under a light microscope), Gram staining and biochemical tests (such as amylase and urease activity, citrate, indole, H<sub>2</sub>S production, methyl red (MR), Voges Proskauer (VP), lactose fermentation, gas formation and triple sugar iron test (TSI)) [40].

### 2.3 Isolation and Identification of Fungi

One ml each from the serially diluted samples was poured into petri plates with Sabouraud dextrose agar (SDA) medium and

incubated at 37 °C for three to four days. The plates were checked for growth of fungal colonies. The colony developed from the infested carapace sample was transferred to SDA slant, stained with Lactophenol cotton blue and observed under light microscope for identification and photographed.

## 3. Results

The carapace of the freshwater crab, *B. cunicularis* was dark brown in colour with tints of lighter shades of brown on the ventral portion (Fig 1A). Infested crabs were observed to have prominent yellow coloured circular lesions measuring < 5 mm diameter, on the ventral carapace (Fig 1B, C) and limb (Fig 1D, E). The lesions were individual and confined to the outer layers of the exoskeleton characterized by limited erosion.

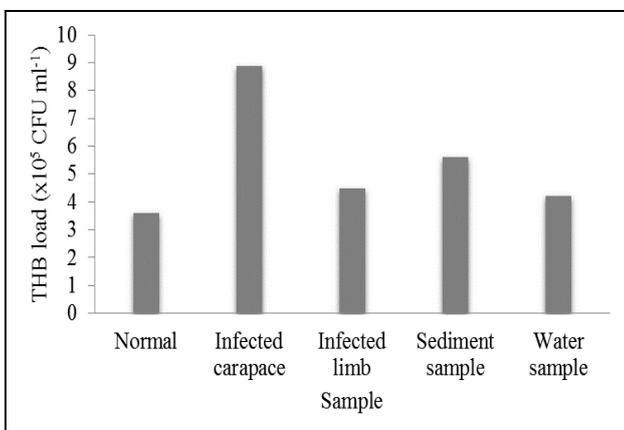




**Fig 1:** A. Carapace of uninfested *Barytelphusa cunicularis*. B, C. Infested carapace. D, E. Infested limb

### 3.1 Enumeration of total heterotrophic bacterial (THB) population

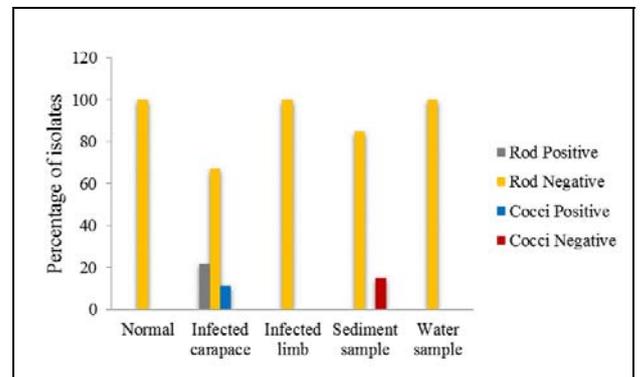
The total number of bacteria in shells of infested and uninfested crabs was estimated after isolation and growth on NA plates. The total heterotrophic bacterial (THB) population in the normal carapace was  $3.6 \times 10^5 \pm 0.18$  CFU ml<sup>-1</sup> and noted to be increased to  $8.9 \times 10^5 \pm 0.02$  CFU ml<sup>-1</sup> in infested carapace. The infested limb region recorded  $4.5 \times 10^5 \pm 0.09$  CFU ml<sup>-1</sup>. The THB load isolated from sediment and water samples were found to be  $5.6 \times 10^5 \pm 0.2$  CFU ml<sup>-1</sup> and  $4.2 \times 10^5 \pm 0.12$  CFU ml<sup>-1</sup> respectively (Fig 2).



**Fig 2:** The total heterotrophic bacterial (THB) load from normal and infested carapace and limb of *Barytelphusa cunicularis*

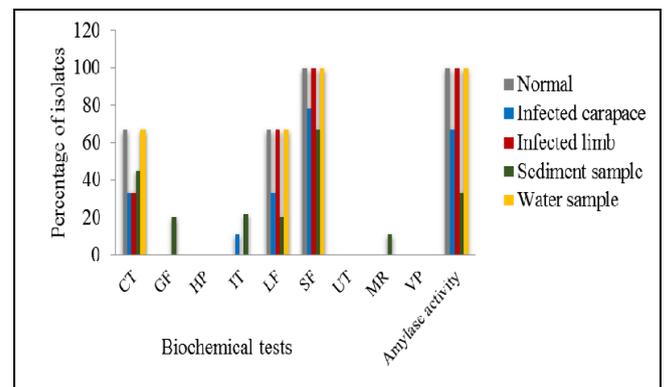
### 3.2 Identification of bacterial isolates

Bacteria were identified based on morphological features, difference in colour of the strain after Gram staining and biochemical tests. All the isolates from normal carapace and water sample in the present investigation were Gram-negative rods; infested carapace showed 67% negative rods, 22% positive rods and 11% positive cocci isolates. Eighty five percent isolates from the sediment sample were negative rods and 15% negative cocci (Fig 3).



**Fig 3:** Percentage of Gram-positive and Gram-negative bacteria isolated from normal and infested carapace and limb of *Barytelphusa cunicularis*

The isolates were analyzed for various biochemical parameters. Amylase activity was positive for 80% of the bacterial isolates. Citrate utilization resulted positive for 40% isolates while 47% showed positive reaction for lactose fermentation. Seven percent of the isolates showed positive reaction for indole test. For the tests, gas formation, H<sub>2</sub>S production, urease activity, MR and VP, none of the isolates yielded positive results. Sugar fermentation test was positive for 87% of isolates. Figure 4 showed comparison and calibration of biochemical response of the bacterial isolates.



**Fig 4:** Percentage of isolates from infested and uninfested carapace and limb of *Barytelphusa cunicularis*, showing positive reaction to various biochemical tests. CT: citrate test, GF: gas formation test, HP: H<sub>2</sub>S production, IT: indole test, LF: lactose fermentation test, SF: triple sugar iron test, UT: urease activity test, MR: methyl red test, VP: Voges Proskauer test.

The results of morphological and biochemical analyses of the infested carapace proved high microbial load with the presence of isolates belonging to nine bacterial genera - *Aeromonas*, *Alcaligenes*, *Bacillus*, *Chromobacterium*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Micrococcus* and *Pseudomonas*. *Pseudomonas* sp. and *E. coli* were found in the

infested limb. *Klebsiella* and *Pseudomonas* were identified from carapace of normal crabs.

### 3.3 Identification of Fungi

After three days of incubation, one fungal colony was observed from infested carapace. The remaining samples did not show any fungal growth. Microscopic observations of the isolated fungi revealed the following characteristics: colourless stripes with smooth walled surface, bi-seriate spherical vesicle serration, globose shape and smooth walled conidial surface. Accordingly the fungus was identified as *Aspergillus* (Fig 5).



**Fig 5:** Light microscopic image of the fungus *Aspergillus* isolated from infested carapace of *Barytelphusa cunicularis*

### 4. Discussion

The chitinous shell of crustaceans not only provides skeletal support, but also protects the soft underlying body. While serving as a physical barrier to the surroundings, the shell is continuously subjected to natural and artificial disturbances rendering them susceptible to a wide variety of pathogens, causing several diseases [41]. Shell diseases have caused great economic losses in aquaculture and fungal infections are second only to bacterial diseases [20]. The current study identified isolates belonging to nine groups of bacteria and one fungal pathogen from the infested carapace and two bacterial genera from the infested limb. The total bacterial load was considerably higher in the infested carapace than the infested limb. Further, normal shells showed limited bacterial flora and no fungal strain was isolated from normal carapace or limb.

In the present investigation, circular, pitted, yellow coloured lesions were observed on the ventral carapace and limb of infested *B. cunicularis*. The lesions were individual, non-coalesced and superficial, suggesting early stages of the disease. Shell disease begins as small raised or pitted puncture or crater-like marks on the ventral carapace or limb [42]. Upon infestation with chitinolytic pathogens, the condition coalesces to form broad irregular lesions with deep necrotic centers, which may or may not penetrate the underlying tissues [17, 43, 44].

The chitinase enzyme produced by chitinolytic bacteria is capable of degrading the carapace chitin causing lesions in crustaceans [45, 46]. These bacteria belong to several genera such as *Vibrio*, *Aeromonas*, *Pseudomonas*, *Alteromonas*, *Flavobacterium*, *Spirillum*, *Moraxella*, *Pasteurella* and *Photobacterium* [47]. The current investigation revealed the presence of nine bacterial genera - *Aeromonas*, *Alcaligenes*, *Bacillus*, *Chromobacterium*, *Enterobacter*, *Escherichia*,

*Klebsiella*, *Micrococcus* and *Pseudomonas* from infested carapace and *Pseudomonas* and *Escherichia coli* from infested limb. Among the bacterial groups identified, *Pseudomonas* and *Aeromonas* were reported to have chitinolytic activity [48]. Lightner and Lewis [49] isolated chitinolytic *Pseudomonas* and *Aeromonas* from lesions of marine shrimps. Although *Pseudomonas* is reported as chitinolytic bacteria, it may be a part of the normal microflora of paddy fields as they were isolated from normal carapace in the present study. Comparable results were also recorded in *C. sapidus*, where *Pseudomonas* species such as *P. alkaligenes*, *P. putrefaciens* and *P. acidovorans* were found in lesioned shells whereas normal shells showed the presence of *P. cepcia* and *P. vesiculans* [50]. In the same species, Noga *et al.* [21] reported the presence of chitinolytic *Aeromonas* species such as *A. sorbia* and *A. punctata* in lesioned shells while normal shells revealed *A. hydrophila* and *A. punctata* suggesting the occurrence of species level variation in bacteria capable of chitinolytic activity. The association of same bacterial genera with both normal and diseased exoskeleton indicates that the microflora found on normal carapace is partly responsible for development of shell lesions [42]. In contrast to the findings of the present investigation, *Klebsiella* was identified along with other chitinolytic bacteria such as *Pseudomonas*, *Aeromonas* and *Vibrio* spp. from lesioned shells of *C. sapidus* [51].

In the current study, bacteria infested carapace lesion showed co-infestation with the fungus *Aspergillus* sp. Fungal pathogens have been reported to cause severe shell lesion epidemics in crustaceans. Studies by Fisher and co-workers [52, 53] identified fungi co-infecting with chitinolytic bacteria in lesioned shells of cultured lobsters. As primary invaders, *Fusarium avenaceum* and *F. solani* caused burn spot disease in the freshwater crayfish, *Astacus astacus* [54]. Generally, pathogenic fungi have been found to grow both on dead and living crustaceans, deriving nutrients from the organic matrix [55].

### 5. Conclusions

Shell lesions in *B. cunicularis* revealed the presence of strains belonging to nine genera of bacteria and one fungal pathogen. Our observations suggest that different combinations of microbes present in the immediate surroundings contribute to the formation of shell lesions. Species level identification of the lesion causing pathogens remains to be determined. Moreover thorough investigations are required to ascertain reasons for the initiation of lesion, spreading mechanism and degradation process of the cuticle and mortality rate among normal population as a result of microbial invasion. Shell disease studies in freshwater crabs, can thus be a useful tool to unravel the relationship between environmental stress conditions and occurrence of the disease.

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