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The hemolymph of healthy *Doclea rissoni* Leach, 1815: A pool for *Vibrionaceae*?

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Original Article

Abstract

As diseases have a great impact on the population dynamics, evolution and immune biology of affected organisms, it is important to understand the modes and mechanisms of interaction among the lower animals and their microbial symbionts in nature function together for survival. To achieve this, it is important to understand the community structure of the native flora of these animals. Spider crabs are well known for their tolerance to various pollutants. Nevertheless, the microbiology of their circulatory systems and their role in disease transmission are unknown. Therefore, the bacteria associated with the hemolymph of a spider crab, *Doclea rissoni* in its natural ecosystem was characterized. Nine healthy crabs were collected from the coastal areas of Kochi, and their hemolymphs were collected. Enumeration of viable bacteria using various media revealed a similar count in all the media (7-10$\times10^2$ CFU/ml). Characterization of representative isolates by conventional microbiological methods and 16S rRNA gene sequencing was followed. Despite using three different media, all isolates belonged to a single family *Vibrionaceae*. There were two genera as *Vibrio* (86.67%) and *Photobacterium* (13.33%). Among *Vibrio*, five species belonging to three different clades were isolated, of which two could not be assigned to any known species. These were classified as novel species (belonging to *Harveyi* clade and, *Brasiliensis* clade) and their description is underway. The order of relative abundance was *V. alginolyticus*>Novel *Vibrio*-II>*P. damselae*>*V. furnissii* / *V. parahaemolyticus* / Novel *Vibrio*-I. Of these, *V. alginolyticus, P. damselae* and *V. furnissii* are implicated in occasional disease outbreaks in humans and animals, posing an increased risk to human economic activity, notably aquaculture practices. Therefore, further investigations are required to find the basis for the mutualistic relationship of vibrios in different crabs.

Keywords: *Doclea rissoni, hemolymph, microbial abundance, microbial symbionts, vibrios*

Introduction

Hemolymph is a critical site for invertebrates as far as host immune response is concerned. Hemolymph was supposed to be "sterile" without proliferating microorganisms in healthy animals, but mounting evidence has established the presence of viable microorganisms in the hemolymph of some aquatic invertebrates (Tubiash *et al*., 1975; Potgieter *et al*., 2015). Such a host-microbe interaction can be obligate for both host and microbe or optional ("facultative") for one of the partners at definite stages of development, yet the exact functions of these hemolymph microorganisms are not well defined (Petersen and Osvatic, 2018). Several hypotheses exist to justify the host-microbe interactions and their physiological role in invertebrates. Schmitt *et al.* (2012) pointed out that such an association can influence the outcome of pathogen infections either by stimulating immunity or by competitive exclusion. Further, the isolation of antimicrobial compounds of bacterial origin from hemolymph has reinforced this hypothesis (Defer *et al*., 2013). However, Garnier *et al.* (2007), Cerf-Bensussan and Gaboriau-Routhiau (2010), observed that, in compromised/ stressed hosts or under unfavourable environmental conditions, these symbionts themselves can act as opportunistic pathogens. As the disease has a great impact on population dynamics, evolution and immune biology of affected organisms (Altizer *et al*., 2003), it is important to understand how modes and mechanisms of interaction among these lower animals and their microbial symbionts in nature function together for their survival. To achieve this, it is important to understand the community structure of the native flora of these animals.

Many reports on the hemolymph microbial abundance in healthy invertebrates showed a range between non-detectable levels and 10^3 CFU/ml, with *Vibrio*, *Acinetobacter* and *Aeromonas* as prevalent groups (Tubiash *et al*., 1975; Sizemore *et al*., 1975, Brandin and Pistole, 1985; Olfasen *et al*., 1993; Ponprateep *et al*., 2012). Recent studies have exposed that temperature had a significant influence on the hemolymph microbial community pattern (Lokmer and Wegner, 2015). However, some specificity in the uptake of bacteria by invertebrate hemolymph exists, with predilections for *Vibrios* as the most abundant genera (Olfasen *et al*., 1993) and their occurrence has been correlated with a rise in environmental temperature (Baross and Liston, 1970). It was interesting that *Vibrios* can utilize different ways of life including free-living, mutualistic, opportunistic or pathogenic forms and thus bacteria of the genus *Vibrio* have been isolated and characterized from healthy as well as moribund invertebrates (Prieur *et al*., 1990; Lacoste *et al*., 2001).

Recent reports on increasing disease load in various marine communities, coupled with predictions of future increases due to climate change, lead the way towards the need for understanding hemolymph-microbe baseline data from a prime immune site like hemolymph in most invertebrates and this will further nourish their health management in culture facilities. Further, a study of this kind will strengthen our knowledge of carrier status invertebrates, particularly with pathogenic organisms in aquaculture/mariculture facilities. In this context, the present study tries to extend our knowledge of the hemolymph microbiota of "Spider crabs" *Doclea rissoni* in its natural ecosystem. The name "Spider crabs" refers to any crab species of the family *Majidae* and, they are widely distributed in the Indo-West Pacific ocean. They are well known for their tolerance to polluted waters and low-oxygen, environments. As most spider crabs are scavengers and inhabit polluted environments, there is every chance of them getting accumulated with various pathogens. Nevertheless, the microbiology of their circulatory system and their role in disease transmission to human and aquatic animals are unknown. Thus present study will provide baseline data on hemolymph microbes of *Doclea rissoni* to throw light on its host-microbe interactions in a natural ecosystem.

Material and methods

Sample collection

A total of nine spider crabs of different sizes (Fig. 1; Table 1) were collected during December 2016-January, 2017 from different locations in Munambam, Kerala. The animals were caught using gill nets, kept in autoclaved seawater and transported to the laboratory within 4 h.

Fig. 1. *Doclea rissoni* under study

Table 1. Morphometrics of crabs under study

Parameter	Animal ID								
	1	2	3	4	5	6	7	8	9
W (g)	42.5	38	48	69.0	72	65	58	60	57
Carapace width (cm)	4.7	4.2	5.3	8.0	8.35	7.54	10.2	10.5	9.7

Isolation and enumeration of bacteria

The hemolymph was collected aseptically using a 23-gauge needle from the ventral sinus of crabs by puncturing through the intersegmental membrane. The puncture site had been swabbed consecutively with a tincture of iodine and 70% alcohol before hemolymph collection. Care was taken to avoid contamination by puncturing into the nearby hepatopancreas and samples supposed to such contamination were discarded. The pooled hemolymph (from 3 crabs) was serially diluted and spread onto Zobell Marine Agar (ZMA), Thiosulphate citrate bile salt sucrose agar (TCBS) and Brain Heart Infusion Agar (BHIA) (Himedia, India) plates in duplicates for assessing bacterial load. Simultaneously, undiluted hemolymph was streaked onto these 3 media for isolation of bacteria. The plates were incubated at 30ºC and were examined for up to 5 days, and morphologically unique colonies were selected for purification. The total viable count was expressed as several colony-forming units (CFU) per ml of hemolymph (Hovda *et al*., 2007). The viable counts of presumptive vibrios (mesophilic *Vibrionaceae* and other closely related *vibrios*) were calculated after an incubation of 48h on TCBS agar (Bolinches *et al*., 1988).

Identification of bacterial isolates

Pure colonies were selected by streaking onto respective media followed by incubation. CTAB method was used for genomic DNA isolation of the pure colonies and characterized by 16S rRNA gene sequencing using universal primers

(Nair *et al.*, 2012). The purity of isolated DNA was verified in 1% agarose gel electrophoresis. On PCR amplification of 16S rRNA gene, an amplicon of ~ 1499 bp was found in all the bacterial isolates. The band size detected in all isolates was consistent as analyzed further by agarose gel electrophoresis. The PCR products were sequenced at a customised DNA sequencing facility (Scigenom, India) using 16S rRNA gene-specific primers. The obtained sequences were then analyzed by NCBI blast for the identification of bacterial isolates. The similarity between 99 and 97% was used as criteria for species and genus assignments and those isolates showing a disparity in the results of biochemical and molecular characterization (16S rRNA gene sequence) were assigned as novel species (Janda and Abbott, 2007). However, by the combined analysis of the results of biochemical and molecular characterization, the bacteria were assigned up to species level (Goodfellow *et al.*, 2012). Representative 16S rRNA gene sequences of each species were then submitted to GenBank (NCBI).

Contribution of hemolymph isolates to disease resistance

The antagonistic activities against common aquatic pathogens were assessed in triplicates using the agar diffusion assay, for which the antagonistic activity was quantified using the size of the growth inhibition zone (in mm) around the spot or the well. (Nair *et al.*, 2012). The proportionate index (PI) was calculated for comparing antagonistic versatility.

Results and discussion

Host-microbe interactions of hemolymph microbiota are supposed to contribute toward pathogen infections either by stimulating immunity or by competitive exclusion. So the present study was carried out to collect baseline information on the hemolymph-microbial association of spider crab *D. rissoni* in its natural ecosystem. Further, the knowledge on the carrier status of these spider crabs to carry organisms having pathogenic potential in culture facilities of other poikilothermic organisms. Spider crabs were chosen in the study as they are widely distributed in the Indo-West Pacific Ocean, known to be scavengers inhabiting polluted environments, thus the chances of getting accumulated with pathogens will be more. The average weight and average length of the spider crab used in the study were 56.61 ± 11.69 g and 7.61 ± 2.39 cm respectively (as mean ±SD).

As an initial step, bacterial load in the hemolymph of *D. rissoni* was assessed in the present study. Even though the earlier circulatory system of healthy animals was thought to be sterile, the presence of bacteria in the hemolymph of *Crustacea*, is currently viewed more as a natural process (Gomez-Gil *et al.*, 2000). But many authors already pointed out that MPN may not reflect the actual predominance of types in the hemolymph since it involves a single medium and the test is qualitative (Stewart *et al.*, 1967; Tubiash *et al.*, 1975). So bacterial load in the hemolymph of *D. rissoni* was quantified in three different media (Table 2). Parallel to the results on other crustaceans, the hemolymph of *D. rissoni* was also found to be nonsterile. The count of bacteria was almost similar in all media (7-11$\times10^2$ CFU/ml). It was noteworthy that the bacterial load observed in the present study was concurrent with observations from many previous reports on invertebrates (Sizemore *et al.*, 1975; Tubiash *et al.*, 1975; Brandin and Pistole, 1875; Olfasen *et al.*, 1993; Ponprateep *et al.*, 2012).

As *Vibrio* spp. was reported as the predominant bacterial type in the hemolymph of blue crabs, and many other invertebrates (Colwell *et al.*, 1975; Davis and Sizemore, 1982; Olfasen *et al.*, 1993), specific enumeration of vibrios was done using TCBS agar. The results obtained (8-9 $\times10^2$ CFU/ml) were comparable with the total bacterial load obtained for the present study. Concurrent to our observations, Colwell *et al.* (1975) report an average of 10^2 CFU/ml vibrios in normal, healthy hemolymph of *Callinectes sapidus.* On the other hand, such comparable results obtained for the total bacterial count in selective (TCBS) and non-selective media in the present study also indicated that most hemolymph bacterial isolates belonged to the family *Vibrionaceae*. However, the study indicated normal hemolymph from apparently healthy spider crab to carry, a moderate vibrio load in it. The exact role of these vibrio-hemolymph associations could not be identified neither it is attributed to infection (Lokner and Wegner, 2015). However, it is proposed that shifts from mutualist- to pathogen-dominated communities can happen with increased bacterial abundance, change in the physiology of host and environmental factors (Ritchie, 2006), which can aggregate the occurrence of infectious diseases (Altizer *et al.,* 2013).

Following enumeration, an assessment of bacterial diversity in the hemolymph was done through culture-dependent methods. A total of 15 morphologically unique colonies could be isolated and when the diverse colonies were characterized, all isolates belonged to a single family *Vibrionaceae* in concordance with the enumeration results.

Table 2. Enumeration in different media

Media	Group I	Group II	Group III
ZMA (cfu/ml)	10×10^2	11×10^2	10×10^2
BHIA(cfu/ml)	9×10^2	9×10^2	7×10^2
TCBS(cfu/ml)	9×10^2	8×10^2	9×10^2

Representative 16*S* rRNA gene sequences submitted to GenBank (NCBI) were assigned with accession numbers (MG077071-MG077076). There were only two genera as *Vibrio* (86.67%) and *Photobacterium* (13.33%). Among *Vibrio* genus, 5 species belonging to 3 different clades were isolated, of which two could not be assigned to any known species. These were classified as novel species. By sequence analysis it was found that novel *Vibrio*-I belonged to *Brasiliensis* clade and novel *Vibrio*- II belonged to *Harveyi* clade and their description is underway. The order of relative abundance was *V. alginolyticus*> Novel *Vibrio*-II> *P. damselae*> *V. furnissii V. parahaemolyticus* / Novel *Vibrio*-I (Fig. 2). Of these, *V. alginolyticus, V. parahemolyticus, P. damselae* and *V. furnissii* are implicated in occasional disease outbreaks in humans and animals, posing increased risk to human economic activity, notably to aquaculture practices.

Vibriosis is recognized as an important marine disease causing significant problems in the development of aquaculture/ mariculture (Chatterjee and Haldar, 2012). However, the epidemiology and role of different marine animals in their infection cycle are unknown. Although *Vibrio* genus is reported as the dominant bacteria among hemolymph microbiota of marine animals (Wang and Wang, 2015), the exclusive presence of single-family *Vibrionacea* in the hemolymph of healthy *Doclea* sp. which lives in a marine environment harbouring diverse cultivable microbes is quite surprising. Maybe this is the reason why Wang and Wang (2015) pointed out the usage of TCBS medium (selective for *Vibrio* sp.) for the characterization of hemolymph microbiota. To increase the efficiency of isolation, in addition to TCBS, we have used 2 more non-selective media in the current research. Additionally, enumeration in all these media also suggested that most hemolymph isolates belonged to *Vibrionaceae*. Therefore, the exclusive presence of *Vibrio* sp. includes known marine pathogens in the hemolymph; where they are coexisting with haemocytes and antimicrobial components, both of which have important roles in phagocytosis and eradication of

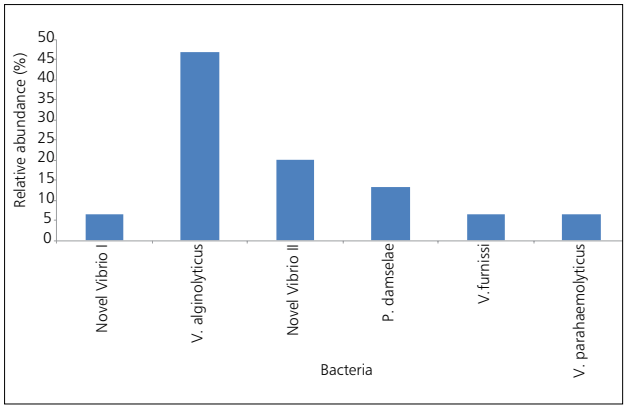

Fig. 2. Relative abundance of bacteria in hemolymph

Table 3. Antagonistic activity of hemolymph isolates

Bacteria	Isolate ID (Zone diameter in mm)				
	109 HL	113 HL	115 HL	117 HL	118 HL
B. cereus	0	5	0	0	0
A. veronii	0	0	0	0	0
A. hydrophila	0	0	0	0	0
P. damselae	0	0	0	0	0
V. alginolyticus	0	0	0	0	0
P. putida	0	0	0	0	0
P. fluorescens	0	0	0	0	0
V. anguillarum	0	0	0	0	0
V. parahemolyticus	0	0	0	0	0
V. vulnificus	0	0	7	4	0
V. harveyi	1	1	6	3	2
PI value	0.091	0.182	0.182	0.182	0.091

bacteria (Fredrick and Ravichandran, 2012) demands further investigations to find the basis for a mutualistic relationship of vibrios in this crab. As the first step of these investigations, we evaluated the antagonistic effects of these isolates against 11 aquatic pathogens. Inhibitory activity was evaluated by measuring the clear zone around culture spots. Out of 15 strains, 5 isolates displayed inhibitory activity against one or more pathogens (Table 3). The highest inhibition was shown against *V. harveyi* (33.33%) followed by *V. vulnificus* (13.33%) and *B. cereus* (6.67%). None of the isolates showed inhibition against the other eight tested pathogens, of which 5 are freshwater, the exposure of which may not come from the tested marine crab. These findings further confirm that some components of crab hemolymph microbiota may have a defensive role against pathogens, and the bacteriostatic effect from symbiotic bacteria and host immune defence may maintain homeostasis.

In conclusion, the results of the present study point out that there is an exclusive presence of *Vibrio* sp. including known marine pathogens in the hemolymph of *D. rissoni* which demands further investigations to find the basis for a mutualistic relationship of vibrios in this crab. The findings also suggest that some components of crab hemolymph microbiota may have a defensive role against pathogens. The findings might contribute to the epidemiology and pathogenesis of *Vibrio*-related diseases. At the same time, with the possibility of *Vibrionales* as a dominant group in culture-dependent methods further investigations are required to find the role of *D. rissoni* in *Vibrio*-related diseases.

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