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Stress responses and vibriosis induced by petroleum hydrocarbons in the penaeid shrimp *Metapenaeus dobsoni* (Miers)

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Abstract

Experiments with juveniles and subadults of the penaeid shrimp *Metapenaeus dobsoni* (Miers) subjected to lethal and sublethal toxicity tests revealed that toxicity of the water accommodated fraction (WAF) of Bombay High Crude were dose-, time-, size- and moult stage-dependant. 13-15% of shrimps (size 30-35 mm) exposed to 1 ppm and 5-8% of shrimps exposed to 4 ppm beyond 18 days in a 30-days moulting pattern experiment developed symptoms of vibriosis. It is significant that the shrimps exposed to 8 ppm and those maintained under control conditions did not show any signs of vibriosis. A chitinoclastic *Vibrio* species was isolated from diseased individuals and Kochs Postulate was successfully proved. Chronic exposure of shrimps to low sublethal doses of petroleum hydrocarbons was found to render them susceptible to vibriosis.

1. Introduction

Metapenaeus dobsoni is the dominant species in penaeid shrimp landings along the southwest coast of India. The juvenile stages of the shrimp are spent in estuarine conditions before they migrate back to the sea. [1,2]. Juveniles and subadults impounded in traditional shrimp farms located in the backwater form the bulk of shrimp culture operations in the region [3]. Petroleum hydrocarbons are omnipresent in the backwaters with the main sources being the Cochin harbour located in the mouth of the estuary and the industrialised belt along the northern regions [4, 5, 6] along with densely populated shores all along the

estuary. The incidence of disease of shrimps, causing very substantial loss to farmers, has become a regular phenomenon [7,8] in the shrimp farms.

Most research work on the effects of petroleum hydrocarbons on crustaceans has been directed at effects on respiration, osmoregulation, moulting, behaviour and chemoreception [9,10]. Lethal and sublethal toxicity of crude and refined oils on several crustaceans have been determined [11]. The effect of hydrocarbons on moulting is a complex phenomenon. Freshly moulted stages of crustaceans have been proved to be most vulnerable to the toxicity of hydrocarbons [12]. The link between shrimp diseases and pollution as one of the major causes has been under investigations for a long period of time [13]. Several workers have proved the link between heavy metal pollution and the incidence of disease and stress in penaeid shrimps [14, 15].

During the course of the investigations on the lethal and sublethal toxicity of Bombay High Crude on *M.dobsoni*, regular incidences of disease in shrimp maintained for over 18 days in toxicant were noticed, which probably indicated stress induced pathogenicity in shrimps.

2. Materials and Methods

2.1 Test Animals

Juveniles and subadults of *M.dobsoni* were collected from shrimp farms in different localities of Vypeen, an island off Kochi, using bag nets and transported to the laboratory where they were acclimated for two weeks in seawater of 20 ± 2 ppt at 22 ±.2°C. Dissolved oxygen was maintained at levels ≥ 3.5 ppm by constant aeration.. Injured and moribund animals were removed during the course of the experiment. They were maintained on a diet of boiled clam and shrimp meat, fed *ad libidum*.

2.2 Preparation of Water Accommodated Fraction of Bombay High Crude

100% Water Accommodated Fraction (WAF) was prepared by churning Bombay High Crude (BHC) with 20 ± 2-ppt seawater in the ratio 1:10 in a 10-litre Perspex tank with a bottom outlet for 14 hours. The WAF was then drained out and its total petroleum hydrocarbon content (TPHC) determined by fluorescent spectrophotometer at 310-360 nm, using standard methods [16] and a BHC standard graph. Fresh WAF was prepared every day for the experiments.

2.3 Toxicological assays

2.3. 1 Lethal toxicity studies

Conventional methods were used to study acute and sublethal responses of the shrimps to PHCs. Intermoult stage juvenile shrimps measuring 20-25 mm, 30-35 mm, 40-45 mm, 50-55mm, 60-65 mm, and subadults measuring 80-85 mm total length were subjected to various dilutions of the WAF in 96-hour LC_{50} tests

using a semi flow through system. The animals were not fed during the experiments. The LC_{50} was computed using Probit analysis [17].

2.3.2 Toxicity tests on freshly moulted shrimps

Juveniles and subadult shrimps were maintained for two weeks in a holding tank until new moon phase, as moulting is more frequent during the new moon. Freshly moulted shrimp were transferred to a second tank and then subjected to experiments in the semi flow through system. Lethal concentrations to bring about 50% mortality of the test population were ascertained and the results were subjected to Probit analysis.

2.3.3 Sublethal toxicity studies

Effects of the sublethal concentrations of PHCs and the moulting frequencies in dosed juveniles of the size range 30-35 mm and 50-55 mm total length in the intermoult stage was studied using a semi flow through system.

2.4 Pathological investigations

Kochs Postulates were tested in four steps to determine the causative pathogen and to identify it upto the generic level. Thereafter the shrimps were challenged with various combinations of PHCs and pathogen concentrations to determine the effect of PHCs on susceptibility to disease.

2.4.1. Isolation of pathogen

Samples of exoskeleton and muscle tissue with the lesion were aseptically excised and impression smear was made on nutrient agar plates supplemented with colloidal chitin.(Peptone . 5 gm, Beef extract 0.3 gm ,colloidal chitin 5 gm ,Sea water 100 ml pH 7).The plates were incubated at 28 C in a Bacteriological Incubator. Chitinoclastic colonies (with halo zone) were isolated onto chitin agar slants (nutrient agar + colloidal chitin). These cultures were streaked onto chitin agar plates for purification and the pure colonies were stocked in chitin agar vials overlaid with sterile liquid paraffin.

2.4.2. Identification of pathogen

Standard methods for identification of the pathogen [18] such as Gram staining, spore staining, mannitol motility tests, marine oxidation- fermentation tests, oxidase test and IMVC test were performed and the pathogen identified to the generic level.

2.4.3. Reinfecting the host with isolate

Suspension of the isolate in sterile saline was prepared by harvesting an eighteen-hour-old slant culture with physiological saline. Optical Density of the suspension was measured at 600 nm Ina Hitachi model 200-20 UV-Visible Spectrophotometer and the corresponding cell number was estimated by direct counting of the gram stained smear preparation using a Nikon Optiphot -2 Research Microscope.

Dilutions ranging from 1x 10^7 to 1x 10 cells per ml were prepared using sterile saline and injected subcutaneously in last abdominal segment of shrimps of the 30-35 mm size range maintained under control conditions in the semi flow through system. Three replicates of each dose were conducted. The course of disease manifestation was followed.

2.4.4. Re isolation of and identification of the pathogen

Isolation of the pathogen from animals in which the disease manifested was performed using the above technique. The pathogen isolated was identified using the standard tests mentioned above and compared with the original isolate of the pathogen.

2.4.5 PHC stress related pathological assay

30-35 mm-sized shrimps were maintained in 8 ppm, 4 ppm and 1 ppm concentrations of PHC for one week. Dilutions containing the pathogen at concentrations from 1x 10^7 to 1x 10^2 cells per ml were prepared using sterile saline and injected into shrimps maintained under control and dosed conditions, replicating the experiment thrice for each dose. The mortality, manifestation of disease and moulting pattern in the injected shrimps were monitored. The results obtained for shrimps under control and dosed conditions were compared by statistical analysis using the test for proportions.

3 Results

3.1 Acute toxicity study results

Probit analysis of the LC_{50} of the various size groups of shrimps subjected to PHCs showed that the size range 15-20 mm total length was most susceptible to PHCs with an LC_{50} of 6 ppm. The shrimp of 20 - 25 mm have an LC_{50} of 7.5 ppm. 30-35 mm, 40-45 mm, 50-55 mm shrimps did not show any significant variation with LC_{50} of 9.2, 9.6 and 9.8 ppm respectively. Subadults of the size range 80-85 mm recorded an LC_{50} of 10.2. There is a marked increase in PHC tolerance between the size range 15-20 mm and between 20=25 mm and 30-35 mm. Mortality in most cases occurred within 48 hours.

3.2 Results of toxicity studies on freshly moulted shrimp

Freshly moulted shrimps of the size ranges 20-25mm, 30-35 mm, 50-55 mm, 80-85 mm have LC_{50} of 2.5 ppm, 3.5 ppm, 3.5 ppm, 4 ppm and 4 ppm respectively. Mortality occurred within the first twelve hours of moulting and no mortality seen beyond 36 hours.

3.3 Effect of sublethal toxicity on moulting

General observations in the moulting pattern made was that moulting of the shrimps the size range 30-35 mm and 50-55 mm under control conditions were

11-12 days and 14 -16 days respectively. Under dosed conditions the suppression of moulting was directly proportional to the increase in dosage of PHCs. The shell of the shrimp formed in dosed conditions was softer than that of the same moult stage shrimps maintained under control conditions.

In the course of the 30-day moulting study, 13-15% of shrimps (size 30-35 mm) exposed to 1 ppm and 5-8% of shrimps exposed to 4 ppm PHCs beyond 18 days developed brown to black lesions on the exoskeleton of the abdominal segments and occasionally on the carapace. Opacity of the musculature of the abdominal segments (Fig.1) and disorientation in swimming were also seen towards beyond 10-14 hours of the manifestation of lesion stages. Afflicted shrimp usually died within 48 hours of external appearance of symptoms. However it was noticed that some shrimps moulted within 12 hours of appearance of lesions. Such shrimps did not develop opacity of muscles. Reappearance of lesions in such shrimps was rare, though in one case lesions reappeared within 24 hours and the shrimp died within 48 hours.. None of these had shown muscular opacity. No disease was noticed in shrimps maintained under control conditions or those in 8 ppm of PHCs.

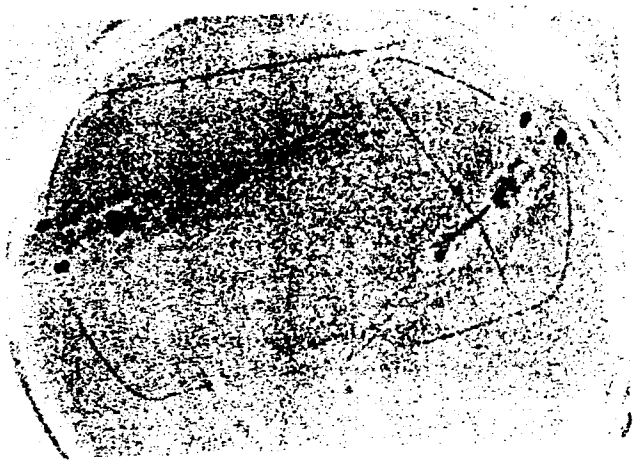

Fig. 1 Lesions caused by *Vibrio* sp. on the abdominal segments of *Metapenaeus dobsoni* when exposed to 1 ppm PHCs for 19 days.

3.4 Results of the pathological assay

The colonies of the pathogen isolated from the infected shrimps were spreading and cream coloured. All the isolates were identical for the morphological and biochemical characteristics tested. The isolates were gram-negative short rods, fermentative, oxidase positive, motile and were identified as vibrios. All the strains were found to be highly chitinoclastic in nature.

The colonies of the pathogen isolated from both the moulting experiment shrimps and the reinfected shrimps isolated as spreading, creamish or grayish coloured, raised, shiny, occasionally translucent, chitin utilizing pure colonies when grown on nutrient agar with colloidal chitin. In both cases the pathogen proved to be a Gram negative short rod which was nonspore forming, catalase test positive, fermentative, and motile and gave positive results for indole production, citrate utilization. It also produced negative results for the Voges-Proskaeur test. The genus of the pathogen was identified as *Vibrio*. Kochs Postulate was proved successfully with the *Vibrio* sp. reinfecting injected shrimps and the reisolation of the pathogen from them.

3.5 Stress effects of PHCs on susceptibility to vibriosis

The results of the PHC stress effects on susceptibility to the Vibrio sp. are given in Table1

Table I: Average percentages of responses of shrimps challenged with *Vibrio* species

Response	Treatment	100000 cells	10000 cells	5000 cells	1000 cells	100 cells
Mortality	Control	100	35	10	0	0
	4 ppm PHC	-	100	100	45	0*
	1 ppm PHC	-	100	100	20	5*
Manifestation	Control	-	90	65	20	0
	4 ppm PHC	-	-	-	60	45
	1 ppm PHC	-	-	-	80	60
Moult	Control	-	0	30	80	100
	4 ppm PHC	-	-	0	10	20
	1 ppm PHC	-	-	-	20	15

$p < 0.01$

* not significant at 1% level

4 Discussion

The present study indicates that the toxicity response of *M.dobsoni* is size dependent with the LC_{50} increasing with size. The margin between LC_{50} of large sized juveniles (60-65 mm) and the LC_{50} of subadults (80-85 mm) is narrow as compared to the difference between LC50s of smaller sizes (20-25 mm and 30-35 mm). Mortality in all doses was restricted to the first 48 hours in the 96-hour LC_{50} tests. However a majority of juveniles (30-35 mm) maintained in the sublethal dose 8 ppm did not survive beyond the 10^{th} day of exposure suggesting that mortality at sublethal doses is more time dependant than in lethal doses. Mortality in sublethal doses may be a cumulative effect of histopathological effects and metabolic disruptions. Capuzzo et. al [19] noted developmental and

energetic abnormalities in lobster larval stages when exposed to PHCs. The LC_{50} of freshly moulted juveniles was much lower than that of intermoult stages of the same size.

The moulting sequence of shrimps exposed to PHCs appears to be complex. In *M.dobsoni* juveniles, the increase in moult cycle was directly proportionate with the dose administered indicating dose dependant moulting rates. The thickness of the carapace has a protective function with crustaceans becoming less vulnerable to PHCs as the carapace progressively hardens after moulting [12]. At the same time juveniles in stages close to moulting were seen to moult prematurely when introduced to even 1 ppm PHCs as also the shrimp maintained under control conditions which were injected with *Vibrio* sp., suggesting that moulting is a stress response dependant on energy availability The energy budget of juveniles maintained in sublethal concentrations may be altered to allocate more energy for body maintenance than for moulting associated processes, leading to the suppression of the latter In the isopod *Mesidotea entomon*, a significant increase in the duration of moults at higher concentrations of hydrocarbons was seen whereas at lower concentrations the effect was stimulation of the onset of subsequent moult. [20].

Vibrios are known aetiological agents responsible for mass mortality of shrimps under cultured and wild conditions [21,22]. Stress caused by the inability of dosed shrimp to moult may be a factor in the *Vibrio* sp. infection. The carapace may be considered a vital disease-resisting barrier in crustaceans and its deterioration provides a route of entry to pathogens [23]. The normal bacterial flora of shrimp includes chitinoclastic bacteria that can become opportunistic pathogens when shrimps are subjected to toxicant stress [14]. Moulting also appears to be a mechanism for elimination of pathogens in shrimps injected with *Vibrio* sp.. A corollary may be that persistent external manifestation of the pathogen takes place in shrimps that are unable to moult and rid themselves of the pathogen creating lesions in the exoskeleton. However no simplistic explanations can be given to susceptibility and immunity of the shrimps, as a host of factors from disruptions in hormonal control to induced cellular and humoral bactericidal activity [23] could be responsible for disease manifestation.

The lack of disease manifestation in shrimps maintained under control conditions and in 8 ppm PHCs in the trial round of injections with *Vibrio* spp. and lower number of manifestations in the lower ranges of pathogen dilutions in 4 ppm dosed shrimps as compared to the 1ppm PHC dosed shrimps suggests the possibility of the pathogen itself being limited by the PHCs. Walker *et.al.* [24] found that PHCs limit the total viable number and populations of chitinolytic bacteria.

The relevance of this study lies in the evidence that stress caused by low sublethal levels of PHC contamination provides an environment conducive for opportunistic pathogens present in concentrations as less as 100 cells/ml to infect juvenile shrimps. This advocates the need for monitoring and controlling low inputs of petroleum hydrocarbons in the estuarine ecosystem.

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