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Characteristics, Pathogenicity and Antibiotic Sensitivity of Bacterial Isolates from White Spot Diseased Shrimp

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Abstract

A bacteriological study was undertaken on white spot diseased shrimp collected from extensive, modified-extensive and semi-intensive ponds located at Visakhapatnam and East Godavari districts of North Coastal Andhra Pradesh. The diseased shrimp exhibited white spots and red discoloration of the body. Four species of bacteria, *V. alginolyticus, V. parahaemolyticus, V. anguillarum* and *Pseudomonas aeruginosa* were isolated from the hemolymph of the diseased shrimp. *V. alginolyticus* was found to be the most dominant and virulent species. All the bacterial isolates showed sensitivity towards oxytetracycline which is a commonly used antibiotic in culture ponds.

Introduction

White spot syndrome (WSS) of penaeid shrimp is often characterized by the development of white spots all over the exoskeleton and red discoloration of the body. It is the most fatal disease affecting shrimp in a culture system in many Asian countries. While the disease is primarily of viral etiology, many species of experimentally virulent, opportunistic, and facultative bacterial pathogens belonging to *Vibrio* sp. invade the diseased shrimp as secondary infections (Lee et al. 1996, Chanrantchakool 1996, Karunasagar et al. 1997). Despite the fact that WSS has been the focus for several investigations dealing with molecular biology, immunology, pathobiology and epizootiology, information pertaining to the bacteriology of the diseased shrimp is still scanty, especially with regard to the species of bacteria involved and their role in the pathological response.

Along the coastal Andhra Pradesh, where shrimp culture is established as a major industry, epizootics due to outbreaks of white spot syndrome have occurred very frequently since their first appearance in shrimp ponds at Nellore

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district in 1993. An epizootiological study of the disease was therefore undertaken from 1994 to 1996 for selected semi-intensive, modified-extensive, and extensive culture ponds situated along the coastal regions of East Godavari and Visakhapatnam districts of Andhra Pradesh. As part of this investigation, a study was conducted on the bacteriology of the white spot diseased shrimp collected. This paper gives an account of the various species of bacteria co-existing with the virus in the diseased shrimp and assesses their potential pathogenic capabilities based on results obtained from experimental infections. Results on the sensitivity of the various bacterial isolates to common antibiotics are likewise presented.

Materials and Methods

Shrimps affected by white spot syndrome were collected from extensive, modified-extensive, and semi-intensive culture ponds located in Kakinada and Visakhapatnam. The affected ponds ranged from 0.8 to 1 ha. in area and 1 to 1.5 m in depth. Commercial pelleted feed was used in all the selected ponds. Salinity was low and varied from 0 to 4 ppt. (Table 1). Water was exchanged every 15 days in semi-intensive ponds while there was no water exchange throughout the crop period in the extensive and modified-extensive ponds.

Altogether 14 moribund shrimps from culture ponds affected with white spot syndrome were brought to the laboratory and subjected to bacteriological analysis. Wet mount preparations of the hepatopancreas and hemolymph were made and examined for the presence of bacteria. Hemolymph was also drawn from the moribund shrimps using a sterile syringe and inoculated into peptone water and incubated at 20°C for 24 hours. Subsequently the samples were diluted serially and inoculated on tryptone soya agar (Himedia, Bombay) plus NaCl (2.5%) using the pour plate technique followed by incubation for 24 to 48 h at 20°C for the development of bacterial colonies. Dominant and morphologically different colonies were selected and transferred onto nutrient agar (containing 2.5% NaCl) plates. Colonies isolated from the diseased shrimp were identified according to the taxonomic schemes of Bergey's Manual of Systematic Bacteriology (Krieg and Holt 1984) and Cowan and Steel's Manual for Medical Bacteria (Barrow and Feltham 1993).

Pathogenicity tests

To fulfill Koch's postulates, experimental infections with the bacterial isolates were carried out on healthy juvenile shrimp weighing approximately 6 g. For this purpose, isolates of different bacterial species (*V. alginolyticus, V. parahaemolyticus, V. anguillarum* and *P. aeruginosa*) were harvested in sterile normal saline solution from 24 h cultures on TSA plates. Different concentrations (0.34×10^3, 0.34×10^4, 0.34×10^5, 0.34×10^6, 0.34×10^7 cfu·gm body weight of shrimp) were injected intramuscularly between the 2nd and 3rd abdominal segments. Fifteen shrimp were injected for each bacterial concentration. Control shrimp were injected with 2% sterile saline solution. Mortalities

were recorded and moribund shrimp were subjected to bacteriological analysis for the reisolation of bacteria.

Susceptibility studies

Sensitivity of the bacterial isolates towards different antibiotics was determined following the disc diffusion method using antibiotic discs (Himedia, Bombay). The diameter of inhibition zone around the antibiotic discs was measured and the extent of sensitivity was assessed using sensitivity standard tables furnished by Himedia, Bombay (1998).

Results

All the fourteen WSS affected shrimp subjected to bacteriological analysis revealed the presence of bacteria, the number of species ranged from 1 to 4 (Table 2). Altogether four species belonging to the genera *Vibrio* (3 spp.) and

Table 1. Details of pond characters, stocking densities and water quality in the ponds affected by white spot syndrome.

Location	Type of culture	No. of prod'n cycles completed	Pond area (ha.)	Source of seed	Stocking density ha^{-1}	Temp. ºC	Salinity ppt	pH	DO
Kakinada	Extensive	8	0.8	Wild	10,000	29	2	6.5	3
Kakinada	Extensive	10	1	Wild	28,570	28	0	8.5	3
Kakinada	Semi-intensive	15	1	Hatchery	105,000	30	1	7.0	4.4
Visakha-patnam	Modified-Extensive	2	1	Hatchery	37,500	28	3	8.2	5.5
Visakha patnam	Modified-Extensive	2	1	Hatchery	34,000	30	4	8.5	5.1

Table 2. Details of bacterial species found in association with the white spot diseased shrimps from culture ponds in the two selected regions

Location	Specimen no.	*V. alginolyticus*	*V. parahaemolyticus*	*V. anguillarum*	*P. aeruginosa*
Kakinada	1	+	+	-	+
	2	+	+	+	+
	3	+	-	+	-
	4	+	-	+	+
	5	+	-	-	-
	6	+	+	+	-
	7	+	+	-	-
Visakhapatnam	8	-	+	+	+
	9	+	+	-	-
	10	-	-	+	-
	11	+	+	-	-
	12	+	+	+	+
	13	-	+	-	-
	14	+	-	+	-

+ = Present; - = Absent

Pseudomonas (1 sp.) were isolated. They were identified as *V. alginolyticus, V. parahaemolyticus, V. anguillarum* and *P. aeruginosa* based on various morphological, physiological and biochemical tests conducted (Table 3).

Bacteria belonging to the genus *Vibrio* were gram negative, motile short rods with a single polar flagellum. They were oxidase and catalase positive and sensitive to 0/129 vibriostatic agent and gave positive reaction for acid production from glucose, nitrate reduction, and glucose fermentation.

Bacterial isolates belonging to the genus *Pseudomonas* were gram negative, motile, oxidase positive, and catalase positive. They produced acid from glucose and reduced nitrate to nitrite. They exhibited growth at 20ºC and 37ºC and produced pyocyanin pigment.

Table 3. Morphological and biochemical characteristics of bacterial isolates from *P. monodon* with white spot disease.

S. no.	Test	*V. alginolyticus*	*V. anguillarum*	*V. parahaemolyticus*	*P. aeruginosa*
1	Gram stain	-	-	-	-
2	Motility	+	+	+	+
3	Flagella	Single	Single	Single	Single
4	Swarming	-	-	-	-
5	Luminescence	-	-	+	+
6	Cytochrome oxidase	+	+	+	+
7	Growth anaerobically	-	-	-	-
8	Catalase	+	+	+	+
9	O/F test	F	F	F	O
10	Gas from glucose	-	-	-	-
11	Nitrate reduction	+	+	+	+
12	Decarboxylation of:				
	a. Arginine	-	+	-	+
	b. Lysine	+	-	+	-
	c. Ornithine	+	-	+	-
13	O/129 sensitivity				
	a. 10 mg	+	-	-	-
	b. 150 mg	+	+	+	-
14	NaCl tolerance :			-	
	a. 0%	-	-	+	-
	b. 1%	+	+	+	+
	c. 2%	+	+	+	+
	d. 3%	+	+	+	+
	e. 4%	+	+	+	+
	f. 6%	+	-	+	-
	g. 7%	+	-	+	-
	h. 8%	-	-	-	-
	i. 10%	-	-	-	-
15	Growth on:				
	a. TCBS agar	+ (Y)	+ (Y)	+ (G)	-
	b. Cetrimide agar	-	-	-	+
	c. MacConkey agar	+	+	+	+
16	Methyl red test	+	+	+	+
17	Voges Proskauer test	+	+	-	-
18	Acid production from Carbohydrates:				
	a. Adonitol	-	-	-	-
	b. Arabinose	-	-	-	-
	c. Cellobiose	-	+	+	-

continued...

Table 3. continued.

S. no.	Test	*V. alginolyticus*	*V. anguillarum*	*V. parahaemolyticus*	*P. aeruginosa*
	d. Dulcitol	-	-	-	-
	e. Fructose	+	+	+	+
	f. Galactose	+	+	-	+
	g. Glucose	+	+	+	+
	h. Inositol	-	-	-	-
	i. Lactose	-	-	-	-
	j. Maltose	+	+	+	+
	k. Mannitol	+	+	+	+
	l. Mannose	+	+	+	+
	m. Melibiose	+	-	+	+
	n. Raffinose	-	-	-	-
	o. Rhamnose	-	-	-	-
	p. Salicin	+	-	-	-
	q. Sorbitol	-	+	+	+
	r. Sucrose	+	+	-	-
	s. Trehalose	+	+	+	+
	t. Xylose	-	-	-	+
19	Indole production	+	+	-	-
20	H2s production	-	-	-	-
21	Citrate utilization	+	+	+	+
22	Gelatinase	-	+	+	+
23	b- galactosidase	-	+	-	-
24	Hydrolysis of starch	+	+	+	-
25	Hydrolysis of urea	+	-	-	+
26	Growth at 4ºC	-	-	-	-
27	Growth at 20 to 30ºC	+	+	+	+
28	Growth at 37ºC	-	-	-	-
29	Growth at 42ºC	-	-	-	-
30	Tween20 hydrolysis	NT	NT	NT	+
31	Tween80 hydrolysis	NT	NT	NT	+

(+ = Positive; - = Negative; NT = Not tested; F = Fermentative; O = Oxidative; Y = Yellow; G = Green).

Among the four species of bacteria associated with the diseased shrimp, *V. alginolyticus* was the most frequently found (78.6% of shrimps), while *P. aeruginosa* occurred rarely. Only two of the 14 shrimps analyzed harbored all four bacterial species while the majority harbored only two (Table 2). In general bacterial diversity was higher among the aged shrimp farms at Kakinada compared to the recently dug farms at Visakhapatnam.

Pathogenicity tests revealed that *V. alginolyticus* was more virulent than the other three bacterial species. The shrimp inoculated with *V. alginolyticus* with a concentration of 0.34×10^4 cfu·gm exhibited 50% mortality within 48 hrs post inoculation. Shrimp injected with *V. parahaemolyticus* at a concentration of 0.34×10^5 cfu·gm and *V. anguillarum* at 0.34×10^4 cfu·g showed 50% mortality in 96 hrs (Table 4).

Drug sensitivity tests revealed that all the *Vibrio* isolates were highly sensitive to chloramphenicol but resistant to ampicillin, cloxacillin and penicillin-G. Isolates of *Pseudomonas aeruginosa* were highly sensitive to ciprofloxacin and norfloxacin. All the isolates were sensitive to oxytetracycline and tetracycline which are commonly used drugs in shrimp culture ponds (Table 5).

Table 4. Mortality rate of *P. monodon* subjected to intramuscular inoculation with bacterial isolates of *Vibrio* and *Pseudomonas* spp. isolated from white spot diseased shrimp.

Bacterial concentration	Mortality (%) of shrimps injected with different bacterial isolates																													
CFU·gm body weight of shrimp	*V. alginolyticus*							*V. anguillarum*							*V. parahaemolyticus*							*P. aeruginosa*						
	Post inoculation time (hours)																													
	4	8	12	24	48	72	96	4	8	12	24	48	72	96	4	8	12	24	48	72	96	4	8	12	24	48	72	96
0.34 x 10^3	0	0	0	0	30	30	50	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.34 x 10^4	0	0	0	20	50	50	60	0	0	0	0	30	40	50	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.34 x 10^5	0	0	100	100	100	100	100	0	0	0	100	100	100	100	0	0	0	10	30	40	50	0	0	10	30	50	50	60
0.34 x 10^6	100	100	100	100	100	100	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.34 x 10^7	100	100	100	100	100	100	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 5. Sensitivity of bacterial isolates of white spot diseased *P. monodon* to various antibiotics.

S. no.	Drug	Sensitivity of bacterial isolates			
		V. alginolyticus	*V. parahaemolyticus*	*V. anguillarum*	*P. aeruginosa*
1	Amoxycillin	-	-	+	-
2	Ampicillin	-	-	-	-
3	Cephadroxil	+++	++	+	-
4	Cefazolin	+	+	-	-
5	Chloramphenicol	+++	+++	+++	-
6	Ciprofloxacin	+++	+++	++	+++
7	Clotrimazole	++	-	-	-
8	Cloxacillin	-	-	-	-
9	Co-Trimoxozole	-	+++	+++	-
10	Erythromycin	++	+	+	-
11	Furazolidin	+	++	+	-
12	Gentamycin	++	-	++	++
13	Metronidazole	-	+	-	-
14	Nitrofurazone	+	+++	+	-
15	Norfloxacin	+++	+++	++	+++
16	Oxytetracycline	++	++	+	+
17	Pefloxacin	+++	+++	++	++
18	Penicillin-G	-	-	-	-
19	Rifampicin	+	+	+	+
20	Streptomycin	+	+	+	+
21	Tetracycline	++	++	+++	++
22	Trimethiprim	++	+++	++	-

+++ = Highly sensitive; ++ = Moderately sensitive; + = Slightly sensitive; - = Resistant.

Discussion

The present study revealed that four species of bacteria namely *Vibrio alginolyticus, V. parahaemolyticus, V. anguillarum* and *Pseudomonas aeruginosa* were present in white spot diseased shrimp. The study of Karunasagar et al. (1997) also reported the presence of four species (*V. alginolyticus, V. cholera, V. mimicus* and *V. harveyi)* in such shrimp. Thus,

only *V. alginolyticus* was common in the two studies. However, opportunistic species may be expected to vary from one geographic area to another and from one pond to another within a farm, depending on the availability of these bacterial pathogens in the external medium.

Pathogenicity studies revealed that *V. alginolyticus* was more virulent than the other three species isolated. Lee et al. (1996) also found *V. alginolyticus* isolated from white spot diseased shrimp from ponds in Taiwan, and its extracellular products (ECPs) were lethal to both *P. monodon* and *P. japonicus*. However, the LC_{50} value of the strain from Taiwan (1.13×10^5 cfu·g) was higher than that of the strain in our study (0.34×10^4 cfu·g). It may be more virulent than the Taiwanese strain.

Vibriosis is a stress induced disease in shrimp. Perhaps WSSV infection reduces the immune competence of shrimp so as to facilitate invasion by a wide range of bacterial pathogens. Environmental stress factors are also known to suppress host resistance to promote viral and bacterial infections. It may be concluded therefore that *Vibrio* infections are mainly derived from environmental causes. Chanratchakool (1996) also observed massive bacterial infections (*Vibrio* spp.) in white spot diseased shrimp with reddish exoskeleton and he considered red discoloration and vibriosis as stress induced diseases.

Although the synergistic effects in dual bacterial and viral infections remained unexplored, there may be a need to minimize bacterial infections through judicious application of antibiotics. All the bacterial isolates identified during the present study showed susceptibility to oxytetracycline which is the most commonly used antibiotic in shrimp culture ponds. Application of antibiotics may reduce the bacterial load but may not affect WSSV, the primary pathogen. Hence it is necessary to prevent the disease through proper management of environmental stress factors.

The role of *Vibrio* bacteria in promoting white spot disease is not well documented, although it is a known fact that they co-exist with the virus in a diseased shrimp. Further studies are required to determine whether these opportunistic pathogens exacerbate the pathological responses of penaeid shrimp to WSSV.

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