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Prevalence of Opportunistic Pathogens in Paddy Cum Shrimp Farms Adjoining Vembanadu Lake, Kerala, India

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Abstract

The prevalence of opportunistic pathogens was monitored in water and sediment samples collected from paddy cum shrimp farms adjoining the Vembanadu Lake during the culture period. The physio-chemical parameters of the environmental samples were also analyzed through the study period so as to find out any possible correlation between the physio-chemical parameters and the prevalence of opportunistic pathogens in the system. *Escherichia coli* isolations were consistently high in all the samples analyzed. High prevalence of opportunistic pathogens such as *Vibrio, Aeromonas* and *Pseudomonas* were also noticed in these paddy cum shrimp farms. However, the environmental parameters did not show any significant variation during the study period and no noticeable disease outbreaks were recorded. *E. coli* strains isolated from the environmental samples were subjected to antimicrobial susceptibility tests and the potential risk posed by the isolates were accessed using multiple antibiotic resistance (MAR) indexing. Results of the antimicrobial susceptibility tests revealed the incidence of multiple antibiotic resistant *E. coli*, originating from high risk sources of contamination. The present findings suggest the need for maintaining the stability of the extensive aquaculture systems and the reduction of the unnecessary usage of antibiotics in them.

Introduction

Traditional paddy cum shrimp farming in '*Pokkali*' fields adjoining the Vembanadu Lake (9º28' & 10º10' N Lat., 78º13' & 76º31' E Long., Kerala, India) is an age-old industry. In these culture operations, the naturally occurring seeds are trapped in tidal impoundments and allowed to grow for short period before they are caught. The shrimps are extensively grown in these

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fields without any additional seed or feed input during November to April-May when the salinity of the feeder canal increases. The paddy cum shrimp culture systems are very productive and are considered as a peaceful coexistence between agriculture and aquaculture. Their capacity to generate proteinaceous food items in addition to paddy is rather commendable. Recently there is an increasing tendency to stock additional seeds, wild and hatchery reared, indiscriminately without any artificial feeding and the environmental parameters are left uncontrolled. Frequent disease outbreaks and resultant crop failures are reported from this area recently. This may because of the stress due to nutritional, crowding and poor environmental parameters, which in turn supports the growth and replication of the opportunistic pathogens in the system.

Unlike their terrestrial counterparts, aquatic animals are more intimately associated with their environment. Any factor that deteriorates the quality of environment might predispose these animals to various types of diseases. Thus, disease may result from bad husbandry and poor water quality conditions, which debilitate the fish and leave them vulnerable to infections by opportunistic pathogens (Austin and Allen 1985). Detailed information about the numbers and type of pathogenic bacteria in aquatic environments is essential, if abnormal conditions such as adverse water quality or the onset of favorable disease conditions are to be recognized and corrected. Emphasis has been placed on specialized groups such as those involved in nutrient cycling or the indicators of water quality (Geldreich 1977), but there are no data on the seasonal microbial shifts which might be expected to occur in natural waters (Allen et al. 1983). Among the various bacterial pathogens reported from the disease conditions in shrimp, *Vibrio* sp. and luminous pseudomonads are widely distributed in marine as well as coastal environments (Lightner 1996). Motile areomonads have been recognized only recently as secondary invaders of various shell diseases in cultured shrimp (Otta et al. 1998).

Agricultural/veterinary use of antimicrobial agents has been much debated. Animals raised for food production naturally should receive treatment for clinical infections. It is estimated that nearly equal tonnage of antimicrobial compounds are used in man and in agriculture worldwide. Published data on the use of antimicrobial agents in fish farming (aquaculture) are very limited. Much remains to be elucidated about the purpose and type of use, as well as about the levels of resistance in bacteria from farmed fish and shellfish. The occurrence of multiple antibiotic resistance among the enteric bacterial species in fish as well as water may make effluents from aquaculture systems of public health significance. If the resistance is plasmid mediated, as has been found commonly in fish pathogens, then there could be a problem associated with the transfer of resistance to other organisms of human/veterinary significance (Toranzo et al. 1983).

In the present study, the prevalence of opportunistic pathogens has been monitored in water and sediment samples collected from the study area. Physiochemical parameters such as temperature, salinity, pH and dissolved oxygen were also recorded throughout the study in order to find out

any variations in them resulting an increased presence of opportunistic pathogens within the system. The antibiotic sensitivity patterns of *Escherichia coli* strains from the environmental samples were also determined and the potential risk posed by the isolates was assessed using multiple antibiotic resistance (MAR) indexing.

Materials and Methods

The present work was carried out for one culture operation (December 2000 to May 2001) in three '*Pokkali*' farms located at Vypeen area adjoining the Vembanadu Lake, having 6ha, 9ha and 16ha of water spread area respectively. Physiochemical as well as bacteriological parameters were analyzed in all the three farms on a monthly basis.

Sample collection

Collection of samples was done on early hours of the day. For bacteriological analysis, water samples were collected in sterile conical flasks from a depth of 0.5m from the surface and sediment samples were aseptically scooped out from the bottom and transferred into sterile polythene bags. For the estimation of the dissolved oxygen content, separate water samples were taken in airtight BOD bottles. All the samples were placed in an ice chest and transported to the laboratory for further analysis and the time between collection and processing was four hours.

Physiochemical analysis of water

Temperature, salinity and pH of water was recorded *in situ*, at the time of sampling using a mercury bulb thermometer, refractometer and a hand pH scan respectively. On reaching the lab, the dissolved oxygen content of water was estimated following modified Winkler's method (Greenberg et al. 1992).

Bacteriological analysis

Samples were analyzed for bacteriological parameters such as total heterotrophic bacterial (THB) load, and most probable number (MPN) of coliforms. Estimation of total heterotrophic bacterial population was done by pour plate technique using Marine agar (Zobell 2216e, Hi media). After incubation at room temperature for 48 - 72 hours plates with colonies ranging from 30 – 300 numbers were selected for counting and the bacterial population was expressed as number of colony forming units (cfu) per milliliter or gram, water or sediment respectively. Representative colonies from each plate were picked, purified and maintained on nutrient agar slants. These isolates were characterized morphologically and physiologically and then identified up to generic level following standard schemes (Buchanan and Gibbons 1979).

MPN of coliforms were determined by three-tube dilution method using lactose broth and incubation at 37ºC. Positive tubes of presumptive MPN test were streaked onto MacConkey agar and eosine methylene blue (EMB) agar and incubated at 37ºC for 24 – 48 hours. Typical colonies were isolated, purified, maintained on nutrient agar slants and confirmed biochemically as *E. coli* using indole, methyl red, voges-proskauer and citrate (IMViC) test.

Antibiotic sensitivity testing

Confirmed *E. coli* strains were screened for resistance towards 12 commonly used antibiotics such as Amikacin (30 mcg), Ampicillin (10 mcg), Chloramphenicol (30 mcg), Ciprofloxacin (30 mcg), Erythromycin (10 mcg), Gentamycin (10 mcg), Nalidixic acid (30 mcg), Oxytetracycline (30 mcg), Penicillin G (10 mcg), Streptomycin (10 mcg), Trimethoprim (30 mcg) and Vancomycin (10 mcg) by standard disc assay technique. The antibiotic discs were dispensed on seeded Mueller-Hinton agar plates and incubated at 37ºC for 24 hours. Based on the diameter of the zone of inhibition, the behavior of each strain towards individual antibiotic was interpreted using standard methods (Bauer et al. 1966). The strains were classified as resistant and sensitive.

Multiple antibiotic resistance (MAR) indexing

MAR indexing when applied to an isolate is calculated by dividing the number of antibiotics to which the isolate is resistant to the total number of antibiotics to which the isolate is exposed. This value is particularly useful in the risk assessment of the isolates. Strains with MAR value higher than 0.2 were considered to have originated from high-risk sources of contamination (Kaspar et al. 1990).

Results and Discussion

The monthly variations in the physiochemical as well as bacteriological parameters of the environmental samples collected from the three sampling stations are given in table 1. The average temperature recorded from the three sampling stations was around 29ºC, while the salinity varied considerably between 19-25 ppt. The frequent changes in tidal amplitude in this area may responsible for these fluctuations in salinity. The results obtained from the analysis of pH and dissolved oxygen of water was within acceptable limits and coincides with the prescribed values, which are conductive for shrimp culture (AQUACOP 1989). The mean variation observed in pH was about 0.9 around the neutral value. A fluctuation in pH around 7 is not a matter of serious concern since brackish water is well buffered against pH changes. Earlier studies in this regard also reported pH fluctuations around neutral during shrimp grow out (Chen and Wang 1990).

Bacteriological analysis of the samples revealed a mean THB load of 7.36 x 10^4 cfu•ml^{-1} in water while in sediment a higher THB load (7.15 x 10^5 cfu•g^{-1}) was observed. The mean variation noted in the THB load of water was 5.7 x 10^4 to 9.4 x 10^4 cfu•ml^{-1} and in sediment the values varied between 6.5 x 10^5 and 7.4 x 10^5 cfu•g^{-1}. The present results are higher than that reported by Nayyarahamed et al. (1995) who obtained THB loads of the order 10^2 to 10^3 cfu•ml^{-1} for water and 10^3 to 10^4 cfu•g^{-1} for sediment from shrimp farms. The total viable heterotrophic bacterial (THB) population in an environment depends on the availability of growth supporting organic matter and micronutrients (Lakshmanaperumalsamy et al. 1981). High counts of THB in sediment samples than water may be due to the abundant micronutrients and organic matter in the sediment. Comparatively higher coliform loads were observed from all the samples. High coliform load in water may due to increased faecal pollution from different sources close to human inhabitation (Fayez et al. 1987). However, in all the samples the coliform load of water was lower than that of sediment. According to Shuval et al. (1973), adsorption and sedimentation tend to remove organisms from suspension and concentrate them in bottom deposits, where they continue an active existence. The observation by Geldreich (1972), that pathogens can survive in the bottom deposits of aquatic environments for several weeks before they die also support our findings.

Characterization of various genera associated with the water and sediment samples revealed that *E. coli* was the predominant organism followed by *Vibrio* and *Pseudomonas* (table 2). The other genera encountered in the present study were *Aeromonas* and *Micrococcus*. An *E. coli* count of < 3•g^{-1} for shrimp and sediment samples from brackishwater farms in Indonesia has been reported earlier (Budisusilowati and Haryani 1995). As there is an increasing amount of evidence as to the direct relationship between human interference and incidence of *E. coli* in water, this indicator organism may

Table 1. Physiochemical and bacteriological parameters of environmental samples from different sampling stations

	Months of Collection	Temperature (°C)	Salinity (ppt)	Dissolved Oxygen (mg•l^{-1})	pH	THB		MPN Index per 100 ml/g	
						Water (cfu•ml^{-1})	Sediment (cfu•g^{-1})	Water	Sediment
Sampling	December	28	22	4.6	6.8	7.8 x 10^4	6.6 x 10^5	430	11000
Station 1	January	31	20	5	6.4	6.7 x 10^4	7.6 x 10^5	2400	11000
	February	30	21	5.02	6.8	6.2 x 10^4	7.9 x 10^5	930	11000
	March	29	22	5.23	7	6.3 x 10^4	7.8 x 10^5	11000	11000
	April	29	23	5.1	7	6.6 x 10^4	7.1 x 10^5	4600	11000
	May	27	22	4.8	7.4	6.9 x 10^4	7.4 x 10^5	11000	11000
Sampling	December	23	20	4.2	6.6	9.6 x 10^4	7.1 x 10^5	4600	11000
Station 2	January	31	23	4.37	6.3	8.8 x 10^4	7.9 x 10^5	4600	11000
	February	30	23	4.87	6.2	9.2 x 10^4	7.4 x 10^5	430	11000
	March	29	24	4.69	6.8	9.7 x 10^4	7.9 x 10^5	11000	11000
	April	29	25	5.3	7.1	9.4 x 10^4	7.3 x 10^5	4600	11000
	May	27	24	5	7.6	9.9 x 10^4	8.1 x 10^5	11000	11000
Sampling	December	28	20	4.8	6.4	5.4 x 10^4	5.9 x 10^5	430	11000
Station 3	January	31	20	4.9	6.6	4.8 x 10^4	5.7 x 10^5	4600	11000
	February	30	19	5	6.1	5.7 x 10^4	6.1 x 10^5	4600	11000
	March	29	21	5.1	6.6	6.2 x 10^4	7.1 x 10^5	11000	11000
	April	29	21	5.9	6.4	5.9 x 10^4	6.8 x 10^5	4600	11000
	May	27	22	5.2	7.2	6.4 x 10^4	7.3 x 10^5	11000	11000

provide an estimate of direct pollution and infection related to water content.

Vibrios are autochthonous to saline water, hence the recovery of *Vibrio* sp. in both water and sediment from brackishwater ponds is quite natural. However, *Vibrio* infections appears to be the most important one in brackish water culture system. A number of *Vibrio* sp. including *V. parahaemolyticus*, *V. harveyi*, *V. vulnificus*, *V. damsella* and *V. alginolyticus* are involved in shrimp diseases. In a tropical area like Cochin, where the water temperature never falls below 20ºC, the distribution of this organism is not much influenced by temperature. The influence of salinity on the survival of *V. parahaemolyticus* is well documented (Martin 1981). However, the salinity of none of the study sites did not showed any significant variation during the period of study.

Pseudomonas was another important genera encountered in water and sediment samples from the study area. They have been frequently reported to cause ulcerative disease in fish (Bullock et al. 1965). Infections caused by pseudomonads are considered to have a very close association with poor environmental conditions. Luminous pseudomonads are reported to have isolated from various shell diseases in shrimp larvae as well as adults (Li and Fleming 1967). In the present study, pigment-producing pseudomonads were isolated from all the samples analyzed.

The other genera encountered in water and sediment samples were *Aeromonas* and *Micrococcus*. *Aeromonas* sp. are ubiquitous in aquatic environments. This microorganism has been consistently isolated from lesion and muscle tissues of infected fishes from India (Kumar et al. 1990) and countries like Thailand (Tonguthai 1985), Malaysia (Wong and Leong 1987), Indonesia (Dana 1987), Myanmar (Roberts et al. 1986) and Sri Lanka (Balasurya 1987). While *Aeromonas hydrophila* is considered as a common inhabitant of

Table 2. Percentage of incidence of opportunistic pathogens encountered in water and sediment from different sampling stations. (N* = 6).

	Genera encountered in Water		Genera encountered in Sediment	
Sampling station 1	*Aeromonas*	(17)**	*Aeromonas*	(33)
	Vibrio	(33)	*Vibrio*	(50)
	Pseudomonas	(33)	*Pseudomonas*	(33)
	E. coli	(50)	*E. coli*	(83)
			Micrococcus	(17)
Sampling station 2	*Aeromonas*	(17)	*Aeromonas*	(33)
	Vibrio	(50)	*Pseudomonas*	(50)
	E. coli	(83)	*E. coli*	(100)
	Micrococcus	(17)	*Micrococcus*	(17)
Sampling station 3	*Aeromonas*	(17)	*Vibrio*	(33)
	Vibrio	(50)	*Pseudomonas*	(17)
	Pseudomonas	(33)	*E. coli*	(83)
	E. coli	(67)	*Micrococcus*	(17)
	Micrococcus	(33)		

*N= Number of water and sediment samples analyzed from each station.
**Figures in parenthesis indicates percentage of incidence of the genera in samples collected from each sampling station.

healthy fish (Hatha et al. 2000) and aquatic system (Kaper et al. 1981). However they have been recognized only recently as secondary invaders in various shell diseases of wild and cultured shrimp (Otta et al. 1998). It is also an established opportunistic pathogen infecting fish under environmental stress (Groberg et al. 1978; Lio-po and Duremdez-Fernandez 1986). Detailed studies conducted on epizootic ulcerative syndrome (EUS) in fishes of inland waters reported that *Micrococcus* sp. in association with *A. hydrophila*, *P. fluorescence* and *E. coli* was isolated and the ulcerated lesions consistently showed the presence of *Micrococcus* sp. (Jingran and Das 1990). However, their pathogenicity in cultured shrimp is not well documented.

In the present study an attempt has been made to assess the potential risk associated with the opportunistic pathogens, using multiple antibiotic resistance (MAR) indexing of a representative organism. Since *E. coli* has been isolated consistently from all the samples, the strains were subjected to antimicrobial susceptibility tests to assess the potential risk posed by them. Table 3 summarizes the percentage of antibiotic resistance of *E. coli* strains isolated in different samples and the MAR indices encountered. All the strains were found to have acquired multiple antibiotic resistance. Hundred percent resistance was recorded against Erythromycin, Penicillin G and Vancomycin. Least resistance (less than 20%) was noticed towards Ciprofloxacin, Gentamycin and Nalidixic acid. The bacterial isolates also showed considerable levels of resistance against Ampicillin (55%) and Oxytetracycline (27%). However in the present study, Amikacin, Chloramphenicol, Streptomycin and Trimethoprim proved to be the most effective antibiotics to which the isolates were sensitive. The results compare favorably with our earlier studies (Hatha et al. 1999), which reports similar resistance patterns of *E. coli* strains isolated from river water. According to Henrik and Moller (2000), the use of antibiotics is the most important factor in amplifying the level of resistance in a given reservoir. In aquaculture operations, the antibiotics are presumed to enter the farming system either through drugs used for prophylactic measures or as incorporated into feed. As can be seen in the results,

Table 3. Percentage of antibiotic resistance of *Escherichia coli* isolates and the MAR indices encountered

Name of the antibiotic	Percentage of resistance (N* = 47)	MAR indices encountered	
Amikacin	0	0.25	(23)**
Ampicillin	55		
Chloramphenicol	0		
Ciprofloxacin	15		
Erythromycin	100	0.33	(40)
Gentamycin	9		
Nalidixic acid	19		
Oxytetracycline	27	0.42	(24)
Penicillin G	100		
Streptomycin	0		
Trimethoprim	0	0.5	(13)
Vancomycin	100		

*N=Total number of isolates.

**Figures in the parenthesis indicate percentage of isolates with given MAR index.

the MAR indices encountered for all the strains in the present study was higher than 0.25. Strains with MAR value higher than 0.2 is considered to have originated from high risk sources of contamination such human where the antibiotics are frequently used.

Evaluating the influence of selected environmental parameters on a particular bacterial population is difficult because of the spatial and temporal heterogeneity of such populations *in situ* and constantly changing conditions in the environment; especially in tidal estuaries (Singleton et al. 1982). The present study highlights the prevalence of opportunistic pathogens in the culture system. However, the stable nature of the physiochemical parameters in the system did not exert much stress on cultured animals and hence no noticeable disease outbreaks was observed during the period of investigation. It is found that any further alternation in the stability of the system could deteriorate the water quality, which may favor the growth and replication of opportunistic pathogens in the system. Furthermore, the MAR indices revealed the high risk nature of isolates from the system, suggesting that the unrestricted and often unnecessary use of antibiotics in aquaculture systems has to be checked.

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