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Ecology of antibiotic resistant vibrios in traditional shrimp farming system (*bhery*) of West Bengal, India

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PEER REVIEW

Peer reviewer

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Comments

This is a good study in which the authors found presence of multiple antibiotic resistant bacteria in traditional shrimp farming system. The outcome is interesting because antibiotic resistant bacteria, emerging out of input based culture system, was detected in those farming areas where antibiotics have never been used.
Details on Page 271

ABSTRACT

Objective: To study the ecology of antibiotic resistant bacteria with emphasis on sucrose negative vibrios in water and sediments samples of traditional shrimp farming system (*bhery*) in West Bengal, India.

Methods: The vibrios were isolated from traditional shrimp farm samples on thiosulphate citrate bile salt sucrose agar and sucrose negative bacterial strains were used as biomarkers to assess the frequency of antibiotic resistance.

Results: The incoming water brought presumptive vibrios ranging from 5.50×10^{1} to 1.00×10^{3} mL in to the *bhery*, and there appeared to build up vibrios in the culture system with days of culture, as there was about 9 fold increase in vibrios. The levels of vibrios were observed to be moderately higher in outlet water and ranged between 4.15×10^{2} and 4.15×10^{3} mL. The counts of vibrios in pond sediment was found to be 1.00×10^{2}-4.90×10^{3} g; while in inlet (2.00×10^{2}-4.20×10^{4} g) and outlet (3.00×10^{2}-6.85×10^{3} g) their levels were observed to be higher than the pond sediment. Thirteen different *Vibrio* species were encountered in traditional shrimp culture system and all vibrios were sensitive to chloramphenicol, followed by ciprofloxacin and gatifloxacin (98.24%), gentamicin (95.61%) and other antibiotics. The multiple antibiotic resistance (MAR), *i.e.*, resistance to at least two antibiotics, was noticed among 43.85% of the sucrose negative vibrios and 41.86% of the sucrose negative non-vibrios. All vibrios *harveyi* strains exhibited MAR. Although no antibiotic was used in the *bhery*, the prevalence of MAR in 44% of the sucrose negative vibrios and non-vibrios is a cause of concern. The MAR index was higher in inlet water and sediment samples. The MAR observed in biomarker strains of pond water and sediment (40%) was comparable to those of inlet samples, thus confirming the fact that incoming water was the major source of antibiotic resistant bacteria.

Conclusions: It seems that the shrimp culture in *bhery* does not favour the proliferation and spread of antibiotic resistant bacteria.

KEYWORDS

Traditional shrimp farming, Penaeus monodon, *Vibrio* spp., Biomarkers, Antibiotic resistance, Mutation frequency, R-plasmid

1. Introduction

In India, since ages, shrimp culture has been followed as a traditional activity. In the early 1990s, shrimp (*Penaeus monodon*) culture witnessed a phenomenal growth as it was a high profit venture. With rapid intensification, serious shrimp disease problems have surfaced. In last 1990s, there were serious problems of viral diseases and environmental

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safety issues, owing to lack of planning and regulation. Various kinds of diseases have resulted in serious losses in cultured shrimp production in many leading shrimp farming countries and increased use of aquaculture drugs[1-3]. Most farmers in West Bengal follow traditional type of shrimp culture in traditional shrimp farms (*bheries*). Although a number of reports are available on the production of shrimp[3-5], research on the microbial ecology in *bheries* is scarce. It is known from an extensive literature that depending on the drug and bacterium, antibiotic resistance can vary geographically and over time[2]. It is, therefore, essential to continue to understand how bacteria respond to antibiotic pressures in various ecological niches especially in coastal shrimp aquaculture systems, where the use of antibiotics is rampant.

A number of studies have been conducted to use changes in fauna of soils to serve as indicators of disturbance. Microbial populations can also be used as biomarkers in a similar way. This type of approach has formed a significant part of risk assessment studies that investigate the release of genetically modified microorganisms in the environment. It involves the use of colony forming units as biomarkers of community structures. Such markers include estimates of species diversity, detection of nutritional groups and antibiotic-resistance profile of populations[6,7]. A combination of culture and analysis of other cellular biomarkers may be useful in future studies on natural microbial populations. Through quantification of bacterial flora of water and sediment and plasmid profiling of antibiotic resistant bacteria, it will become clear if traditional shrimp aquaculture is facing a real threat from the use of antibiotics. Therefore, the present study investigated the ecology of antibiotic resistant bacteria with special reference to sucrose negative vibrios in water and sediments samples of traditional shrimp farming system in West Bengal, India.

2. Materials and methods

2.1. Sampling area and culture details

A traditional brackishwater shrimp culture system, locally called as *bhery*, in Malancha (Lat 22° N; Long 88°45′ E), South 24 Parganas district, West Bengal, India culturing *Penaeus monodon* and brackishwater fishes was selected to assess the microbial ecology for one crop period of 9 months between March 2011 and November 2011. The source water for the *bhery* was drawn from the Bidhyadhari River. The species cultured include penaeids such as *Penaeus monodon*, *Fenneropenaeus indicus* and *Metapenaeus* sp. and finfishes such as *Liza parsia*, *Lates calcarifer*, *Oreochromis mossambicus* and *Mystus* sp. The selected *bhery* with the total area of 5.33 ha and water spread area of 4.67 ha was a seasonal one. The depth of the *bhery* was 1.5-2.0 m. The sampled *bhery* was surrounded by other *bheries* and few brick kilns. After every culture, the *bhery* was completely drained and allowed to dry for a period of about 10 days. The sludge was removed up to a depth of 30 cm and the *bhery* was ploughed by a tractor when completely dried. The inorganic chemicals used were agricultural lime (214 kg/ha), bleaching powder (10 kg/ha) and thydon oil (an insect killer @ 1.3 L/ha) for treating pond sediment. Water was let in to the *bhery* after a month of treatment. Total duration of *bhery* preparation was about 2 months. Dry cow dung (1 070 kg/ha), mahua oil cake (150-200 kg/ha) and single super phosphate (642 kg/ha) were used initially. The fish of weight 4-5 g and shrimp post-larvae stocked were mainly wild collections from Midnapore and Kakdwip regions of West Bengal. Hatchery raised shrimp seeds of *Penaeus monodon* procured from Andhra Pradesh, India were also stocked. Once the desirable primary productivity was achieved, the seeds were stocked repeatedly at fifteen days interval. The stocking densities of the shrimp and fish were approximately 55 000 post-larvae/ha and 5 500/ha, respectively. The salinity of the pond water was in the range of 1.00-18.50 ppt during the culture period. No artificial feed was given to the shrimp and fish and they sustained theirselves on natural foods. No chemicals and antibiotics were used in the *bhery* during the culture operation. Repeated harvesting was done periodically on attaining marketable size.

2.2. Enumeration and identification of Vibrios

The water samples from the column region of pond, inlet and outlet were collected in sterile 250 mL polypropylene bottles. The sediment samples were collected aseptically using sterile plastic containers from pond, inlet and outlet and transferred to sterile polythene bags separately. The pond sediment samples were collected from four places, *viz*., nearer to inlet and outlet and two different places of *bhery* along the sides. All the samples were placed in an insulated container and brought to the laboratory within 3 h of collection. Before bacteriological analysis, the water samples were mixed thoroughly. The pond sediment samples taken from four different places were pooled together aseptically and mixed thoroughly. Ten fold serial dilutions of water and sediment samples were done with sterile diluent containing 1% (w/v) sodium chloride. Appropriate dilutions were inoculated onto thiosulphate citrate bile salt sucrose (TCBS) agar plates for the enumeration of presumptive *Vibrio* counts (PVC) by spread plate technique. The plates were incubated at (30±2) °C for 24 h, noted colony forming units and the counts per g and mL of the samples were calculated[5]. The green colour colonies (sucrose negative), based on dominance and distinct colony morphology, were picked from the TCBS agar, purified by repeated streaking on tryptic soy agar with 1% (w/v) sodium chloride (TSAS) and then maintained on TSAS slants. These sucrose negative bacterial strains were used as biomarkers to assess the frequency of antibiotic resistance as they were reported to be the dominant flora of shrimp guts and majority of them are shrimp pathogens[1,8]. The taxonomic keys proposed by Alsina and Blanch were followed for the identification of vibrios[9,10].

2.3. Antibiotic sensitivity assay

A total of 157 sucrose negative bacterial isolates comprising vibrios (n=114) and non-vibrios (n=43) were tested for their antibiotic sensitivity by agar disc diffusion technique on Mueller Hinton agar with 1% sodium chloride (MHA)[11]. The antibiotic impregnated discs (HiMedia, Mumbai) and their concentration

used include amoxyclav (30 μg), chloramphenicol (30 μg), clindamycin (2 μg), ciprofloxacin (5 μg), co-trimoxazole (25 μg), erythromycin (15 μg), gatifloxacin (5 μg), gentamicin (10 μg), nitrofurantoin (300 μg), oxytetracycline (30 μg), sulfafurazole (300 μg) and vancomycin (30 μg). Young cultures of bacteria (20 h old) from TSAS slants were inoculated individually in Mueller Hinton broth with 1% sodium chloride, incubated for 10-12h at (30±2) °C and swabbed on to MHA plates. Antibiotic impregnated discs were placed aseptically on to the seeded agar plates. The plates were then incubated for 24 h at (30±2) °C, measured the zone of inhibition in mm and interpreted the sensitivity of bacteria.

2.4. Mutation frequency

Three chloramphenicol sensitive strains, *viz.*, *Vibrio hollisae* (*V. hollisae*) (strains M76, M78) and *Vibrio campbellii* (*V. campbellii*) M110 isolated from water and sediment samples were used for determining the mutants resistant to chloramphenicol. Young cultures of these strains grown in 300 mL tryptic soy broth with 1% sodium chloride were harvested and washed twice with sterile saline (1% sodium chloride) by centrifugation at 6000 r/min for 10 min each. The deposits were then resuspended in 5 mL sterile saline to a concentration of about 1010 cells/mL and used immediately. Mutation frequency was estimated by the method of Barnes *et al*[12]. The seeded agar plates with or without chloramphenicol (25 μg/mL) were incubated at (30±2) °C for 5-7 d. The number of colonies on the plates was counted and mutation frequency calculated.

2.5. Plasmid DNA isolation

Plasmid DNA of oxytetracycline resistant *V. campbellii* M110 was isolated using alkali lysis method[13]. Plasmids were isolated using plasmid isolation kit (Bangalore Genei Pvt Ltd, India) following the manufacturer's instructions. The plasmids were checked by agarose gel electrophoresis through 1% agarose gel.

2.6. Statistical analysis

ANOVA was followed to test the level of significance among the bacteriological characteristics, after log transformation. The critical difference was calculated to examine which source differed significantly. Simple correlation was used to correlate the biotic parameters. *Chi*-square test was followed to observe the significant difference between the multiple antibiotic resistance of vibrios and non-vibrios[14].

3. Results

The presumptive *Vibrio* counts (PVCs) of water and sediment samples from the inlet, pond and outlet of traditional shrimp culture system and the correlation coefficient among the bacterial flora are presented in Tables 1 and 2, respectively.

Table 1

The presumptive *Vibrio* counts of water and sediment samples from the inlet, pond and outlet of traditional shrimp culture system (*bhery*).

Days of culture	Water			Sediment		
	PVC/mL	Suc-V/mL	Suc+V/mL	PVC/g	Suc-V/g	Suc+V/g
Inlet						
0	1.00×10^{3}	7.00×10^{2}	3.00×10^{2}	4.20×10^{4}	$1.7\ 0\times10^{4}$	2.50×10^{4}
30	5.50×10^{2}	3.00×10^{2}	2.50×10^{2}	1.23×10^{4}	5.30×10^{3}	7.00×10^{3}
60	7.50×10^{2}	3.00×10^{2}	4.50×10^{2}	1.03×10^{4}	7.95×10^{3}	2.30×10^{3}
90	4.15×10^{2}	1.80×10^{2}	2.35×10^{2}	4.60×10^{3}	2.60×10^{3}	2.00×10^{3}
120	6.50×10^{1}	0.50×10^{1}	6.00×10^{1}	2.00×10^{2}	0.50×10^{2}	1.50×10^{2}
150	5.50×10^{1}	2.00×10^{1}	3.50×10^{1}	3.50×10^{2}	2.50×10^{2}	1.00×10^{2}
180	2.70×10^{2}	1.55×10^{2}	1.15×10^{2}	6.00×10^{2}	3.00×10^{2}	3.00×10^{2}
210	8.00×10^{1}	2.50×10^{1}	5.50×10^{1}	1.90×10^{3}	$<1.00\times10^{2}$	1.90×10^{3}
240	4.30×10^{2}	1.10×10^{2}	3.20×10^{2}	2.05×10^{3}	1.30×10^{3}	7.50×10^{2}
Pond						
0	2.60×10^{2}	1.20×10^{2}	1.40×10^{2}	2.80×10^{3}	1.30×10^{3}	1.50×10^{3}
30	1.00×10^{2}	$<1.00\times10^{2}$	1.00×10^{2}	1.00×10^{2}	1.00×10^{2}	$<1.00\times10^{2}$
60	2.50×10^{2}	1.00×10^{2}	1.50×10^{2}	1.65×10^{3}	1.35×10^{3}	3.00×10^{2}
90	1.04×10^{3}	3.60×10^{2}	6.80×10^{2}	4.90×10^{3}	8.00×10^{2}	4.10×10^{3}
120	9.30×10^{3}	$<1.00\times10^{2}$	9.30×10^{3}	1.00×10^{2}	0.50×10^{2}	0.50×10^{2}
150	2.80×10^{2}	3.00×10^{1}	2.50×10^{2}	1.00×10^{2}	0.50×10^{2}	0.50×10^{2}
180	3.00×10^{2}	5.50×10^{1}	2.45×10^{2}	2.00×10^{2}	1.00×10^{2}	1.00×10^{2}
210	4.90×10^{2}	5.50×10^{1}	4.35×10^{2}	3.50×10^{2}	3.50×10^{2}	$<1.00\times10^{2}$
240	1.95×10^{2}	4.00×10^{1}	1.25×10^{2}	3.00×10^{3}	1.95×10^{3}	1.05×10^{3}
Outlet						
0	2.50×10^{3}	2.00×10^{3}	5.00×10^{2}	3.40×10^{3}	1.50×10^{3}	1.90×10^{3}
30	1.00×10^{3}	7.50×10^{2}	2.50×10^{2}	2.25×10^{3}	1.20×10^{3}	1.05×10^{3}
60	1.00×10^{3}	5.50×10^{2}	4.50×10^{2}	1.65×10^{3}	1.30×10^{3}	3.50×10^{2}
90	4.60×10^{2}	1.00×10^{2}	3.60×10^{2}	1.55×10^{3}	8.50×10^{2}	7.00×10^{2}
120	5.95×10^{2}	1.00×10^{2}	4.95×10^{2}	1.25×10^{3}	6.85×10^{2}	5.65×10^{2}
150	4.15×10^{3}	1.65×10^{3}	2.50×10^{3}	5.65×10^{3}	3.90×10^{3}	1.75×10^{3}
180	5.65×10^{2}	4.00×10^{1}	5.25×10^{2}	3.00×10^{2}	1.00×10^{2}	2.00×10^{2}
210	4.15×10^{2}	1.35×10^{2}	2.80×10^{2}	6.85×10^{3}	3.80×10^{3}	3.05×10^{3}
240	6.60×10^{2}	4.50×10^{2}	2.10×10^{2}	3.05×10^{3}	1.65×10^{3}	1.40×10^{3}

Suc+V: Sucrose positive presumptive *Vibrios*, Suc-V: Sucrose negative presumptive *Vibrios*.

Table 2

Correlation coefficient (*r*) among the bacterial flora of *bhery* water and sediment.

Parameters	Water			Sediment		
	PVC	Suc-V/mL	Suc+V/mL	PVC	Suc-V/g	Suc+V/g
Inlet						
PVC	1			1		
Suc-	0.946^{**}	1		0.876^{**}	1	
Suc+	0.961^{**}	0.841^{**}	1	0.966^{**}	0.747^{*}	1
Pond						
PVC	1			1		
Suc-	0.181	1		0.940^{**}	1	
Suc+	0.986^{**}	0.081	1	0.947^{**}	0.804^{**}	1
Outlet						
PVC	1			1		
Suc-	0.848^{**}	1		0.982^{**}	1	
Suc+	0.730^{*}	0.312	1	0.935^{**}	0.853^{**}	1

*:$P<0.05$), **: $P<0.01$; Suc+V: Sucrose positive presumptive *Vibrios*, Suc-V: Sucrose negative presumptive *Vibrios*.

The PVCs of inlet water, pond water and outlet water were recorded in the range of 5.50×10^{1}-1.00×10^{3}/mL, 1.00×10^{2}-9.30×10^{3}/mL and 4.15×10^{2}-4.15×10^{3}/mL, respectively. The sucrose negative presumptive vibrios in inlet water, pond water and outlet water were found to vary between 0.50×10^{1} and $7.00\times$

10^2/mL, $3.00\times10^1$and 3.60×10^2/mL, 4.00×10^1 and 2.00×10^3/mL, respectively. Likewise the sucrose positive presumptive vibrios in inlet water, pond water and outlet water ranged from 3.50×10^1–4.50×10^2/mL, 1.00×10^2–9.30×10^3/mL and 4.00×10^1–2.00×10^3/mL, respectively. The PVCs of sediment samples were in the range of 1.00×10^2 – 4.20×10^4/g. Of the 157 sucrose negative isolates from water and sediment, 114 were vibrios and the remaining 43 were non–vibrios, comprising 27.39% of sucrose negative isolates on TCBS agar (Table 3).

All vibrios were sensitive to chloramphenicol followed by ciprofloxacin and gatifloxacin (98.24%), gentamicin (95.61%), oxytetracycline (87.71%), nitrofurantoin and co–trimoxazole (83.33%), erythromycin (78.94%), amoxyclav (75.43%), sulfafurazole (58.78%), vancomycin (48.24%) and clindamycin (25.43%). About 97.68% of the non–vibrios were sensitive to chloramphenicol and ciprofloxacin followed by gatifloxacin and gentamicin (95.34%), erythromycin and nitrofurantoin (83.72%), co–trimoxazole (76.74%), amoxyclav (72.09%), oxytetracycline and sulfafurazole (67.44%), vancomycin (51.17%) and clindamycin (34.89%).

Table 3

Antibiotic sensitivity of bacterial flora (n=157) of *bhery*.

Antibiotics (μg)	Number of isolates (%)					
	Sucrose negative vibrios (n=114)			Sucrose negative non–vibrios (n=43)		
	R	I	S	R	I	S
Amoxyclav (30)	10 (8.78)	18 (15.79)	86 (75.43)	5 (11.62)	7 (16.28)	31 (72.09)
Chloramphenicol(30)	0 (0)	0 (0)	114 (100)	0 (0)	1 (2.32)	42 (97.68)
Ciprofloxacin (5)	2 (1.76)	0 (0)	112 (98.24)	0 (0)	1 (2.32)	42 (97.68)
Clindamycin (2)	63 (55.27)	22 (19.30)	29 (25.43)	20 (46.51)	8 (18.60)	15 (34.89)
Co–trimoxazole (25)	9 (7.90)	10 (8.78)	95 (83.33)	3 (6.98)	7 (16.28)	33 (76.74)
Erythromycin (15)	6 (5.27)	18 (15.79)	90 (78.94)	3 (6.98)	4 (9.30)	36 (83.72)
Gatifloxacin (5)	0 (0.00)	2 (1.76)	112 (98.24)	0 (0)	2 (4.66)	41 (95.34)
Gentamicin (10)	2 (1.76)	3 (2.63)	109 (95.61)	2 (4.66)	0 (0)	41 (95.34)
Nitrofurantoin (300)	6 (5.27)	13 (11.40)	95 (83.33)	3 (6.98)	4 (9.30)	36 (83.72)
Oxytetracycline (30)	8 (7.01)	6 (5.27)	100 (87.71)	8 (18.60)	6 (13.96)	29 (67.44)
Sulfafurazole (300)	16 (14.03)	31 (27.20)	67 (58.78)	7 (16.28)	7 (16.28)	29 (67.44)
Vancomycin (30)	35 (30.70)	24 (21.06)	55 (48.24)	13 (30.23)	8 (18.60)	22 (51.17)

R: Resistant, I: Intermediate, S: Sensitive.

Table 4

Species–wise particulars of antibiotic resistance in bacterial flora of *bhery*.

Species	Inlet				Pond				Outlet			
	Water (n=28)		Sediment (n=26)		Water (n=15)		Sediment (n=20)		Water (n=33)		Sediment (n=35)	
	Prevalence (%)	MAR %	Prevalence (%)	MAR %	Prevalence (%)	MAR %	Prevalence (%)	MAR %	Prevalence (%)	MAR %	Prevalence (%)	MAR %
Listonella anguillarum	–	–	–	–	–	–	–	–	3.03 (1)	3.03 (1)	2.86 (1)	–
V. campbellii	25.00 (7)	3.58 (1)	26.92 (7)	7.70 (2)	20.00 (3)	–	10.00 (2)	10.00 (2)	18.19 (6)	9.10 (3)	5.71 (2)	2.86 (1)
Vibrio fluvialis	–	–	7.70 (2)	3.84 (1)	–	–	–	–	12.12 (4)	3.03 (1)	2.86 (1)	–
Vibrio furnissii	3.58 (1)	3.58 (1)	–	–	–	–	–	–	6.07 (2)	–	2.86 (1)	2.86 (1)
Vibrio harveyi	–	–	7.70 (2)	7.70 (2)	–	–	–	–	–	–	2.86 (1)	2.86 (1)
V. hollisae	3.58 (1)	3.58 (1)	11.53 (3)	7.70 (2)	6.67 (1)	–	10.00 (2)	–	–	–	14.29 (5)	8.58 (3)
Vibrio metschnikovii	3.58 (1)	3.58 (1)	3.84 (1)	–	–	–	–	–	–	–	–	–
Vibrio minicus	14.29 (4)	–	–	–	13.33 (2)	6.67 (1)	10.00 (2)	10.00 (2)	12.12 (4)	3.03 (1)	20.00 (7)	11.42 (4)
Vibrio parahaemolyticus	3.58 (1)	–	3.84 (1)	3.84 (1)	–	–	–	–	–	–	–	–
Vibrio splendidus II	14.29 (4)	3.58 (1)	11.53 (3)	11.53 (3)	–	–	5.00 (1)	–	3.03 (1)	–	–	–
Vibrio vulnificus	–	–	–	–	6.67 (1)	6.67 (1)	–	–	12.12 (4)	6.07 (2)	–	–
Unidentified *Vibrio* spp.	3.58 (1)	–	3.84 (1)	–	–	–	15.00 (3)	–	–	–	2.86 (1)	2.86 (1)
Plesiomonas shigelloides	10.71 (3)	10.71 (3)	3.84 (1)	3.84 (1)	13.33 (2)	6.67 (1)	15.00 (3)	10.00 (2)	9.10 (3)	–	11.42 (4)	5.71 (2)
Non–vibrios	17.86 (5)	7.14 (2)	19.23 (5)	7.70 (2)	40.00 (6)	20.00 (3)	35.00 (7)	10.00 (2)	24.24 (8)	12.12 (4)	34.29 (12)	14.29 (5)

MAR: Multiple antibiotic resistance; –: No isolates.

Table 5

Multiple antibiotic resistance index among the bacterial flora of *bhery*.

Species	MAR index range	% of isolates with MAR index						MAR%
		0	0.09	0.17	0.25	0.33	0.41	
Listonella anguillarum (n=2)	0.00–0.25	50.00	–	–	50.00	–	–	50.00
Vibrio campbellii (n=27)	0.00–0.33	22.22	40.74	22.22	7.40	7.40	–	37.02
Vibrio fluvialis (n=7)	0.00–0.17	28.58	42.85	28.58	–	–	–	28.58
Vibrio furnissii (n=4)	0.00–0.17	25.00	25.00	25.00	–	25.00	–	50.00
Vibrio harveyi (n=3)	0.17–0.33	–	–	66.67	–	33.33	–	100
Vibrio hollisae (n=12)	0.00–0.33	16.67	41.67	–	25.00	16.67	–	41.67
Vibrio metschnikovii (n=2)	0.00–0.33	50.00	–	–	–	50.00	–	50.00
Vibrio minicus (n=19)	0.00–0.17	42.10	15.80	42.10	–	–	–	42.10
Vibrio parahaemolyticus (n=2)	0.09–0.33	–	50.00	–	–	50.00	–	50.00
Vibrio splendidus II (n=9)	0.00–0.17	44.44	22.22	33.33	–	–	–	33.33
Vibrio vulnificus (n=5)	0.00–0.17	20.00	20.00	60.00	–	–	–	60.00
Unidentified *Vibrio* spp. (n=6)	0.00–0.17	33.33	50.00	16.67	–	–	–	16.67
Plesiomonas shigelloides (n=16)	0.00–0.25	31.25	12.50	50.00	6.25	–	–	56.25
Non–*Vibrios* (n=43)	0.00–0.41	34.89	23.25	13.95	9.30	13.95	4.66	41.86
Total (n=157)	0.00–0.41	30.58	26.75	25.48	7.00	8.91	1.28	42.67

–: No data.

Table 6
Multiple antibiotic resistance index among the sucrose negative bacterial flora of different sources.

Sources	MAR index	% of isolates with MAR index						MAR %
	range	0	0.09	0.17	0.25	0.33	0.41	
Inlet water (*n*=28)	0–0.41	39.28	28.58	21.42	–	7.14	3.58	32.14
Inlet sediment (*n*=26)	0–0.33	19.23	26.93	23.08	11.53	19.23	–	53.84
Pond water (*n*=15)	0–0.33	26.66	33.34	26.66	6.67	6.67	–	40.00
Pond sediment (*n*=20)	0–0.17	25.00	35.00	40.00	–	–	–	40.00
Outlet water (*n*=33)	0–0.25	39.39	24.24	27.27	9.10	–	–	36.37
Outlet sediment (*n*=35)	0–0.41	28.58	20.00	20.00	11.42	17.14	2.86	51.42
Total (*n*=157)	0–0.41	30.58	26.75	25.48	7.00	8.91	1.28	42.67

–: No data.

Table 7
Antibiotic resistance profiles of sucrose negative *Vibrios* and non–*Vibrios* of *bhery*.

Resistant phenotype	Sucrose negative *Vibrios* (*n*=114)						Sucrose negative non–*Vibrios* (*n*=43)					
	IW	IS	PW	PS	OW	OS	IW	IS	PW	PS	OW	OS
None	9	3	3	2	10	6	2	2	1	3	3	4
A		1										
L	3	3	1	3	5	4	1		1			3
S	1											
T				2	1					2	1	
V	3	2	2		1			1	1			
A,L	2		1									
A,S	1					1						
L,N	1											
L,O						1						
L,S	1			3		1					2	
L,V	1	5	1	3	7	2			2	1		
N,O										1		
S,T		1				2						
A,L,O												1
A,L,V			1			1						
L,N,O		2				1						1
L,O,V		1										
L,S,V					1						1	
O,S,V											1	
A,G,L,V		1				1	1					1
A,L,O,V												1
E,F,S,T	1					1						
E,L,N,O						2						
E,L,O,V		1										
E,L,S,T								1				
E,L,S,V		1						1				
L,O,S,V									1			
A,E,L,N,O							1					
E,L,O,S,V												1

A: amoxyclav (30 μg); B: gatifloxacin (5μg); C: chloramphenicol (30μg); E: erythromycin (15μg); F: ciprofloxacin (5μg); G: gentamicin (10μg); L: clindamycin (2μg); N: nitrofurantoin (300μg); O: oxytetracycline (30μg); S: sulfafurazole (300 μg); T: co–trimoxazole (25 μg); V: vancomycin (30 μg) IW: inlet water IS: inlet sediment, PW: pond water, PS: pond sediment, OW: outlet water, OS: outlet sediment.

Thirteen different species such as *Listonella anguillarum*, *Plesiomonas shigelloides*, *V. campbellii*, *Vibrio fluvialis*, *Vibrio furnissii*, *Vibrio harveyi (V. harveyi)*, *V. hollisae*, *Vibrio metschnikovii*, *Vibrio minicus*, *Vibrio parahaemolyticus Vibrio splendidus* II, *Vibrio vulnificus* and unidentified *Vibrio* spp. were encountered in the samples taken from the inlet water, pond water, outlet water, inlet sediment, pond sediment and outlet sediment. The maximum multiple antibiotic resistance (MAR) up to 100% was observed in *V. harveyi* followed by *Vibrio vulnificus* (60%), *Plesiomonas shigelloides* (56.25%), *Listonella anguillarum*, *Vibrio furnissii*, *Vibrio metschnikovii*, *Vibrio parahaemolyticus* (50% each). The maximum MAR was observed in inlet sediment (53.84%) followed by outlet sediment (51.42%). The MAR observed in pond water and pond sediment was 40%. The MAR index range was 0–0.41 (Tables 4–6).

The antibiogram data from 157 bacterial strains yielded 29 different resistance profiles, *i.e.*, 23 and 18 resistance profiles among sucrose negative vibrios and non–vibrios, respectively (Table 7). The mutation frequency of *V. hollisae* (M76 and M78) and *V. campbellii* M110 strains to chloramphenicol (25 μg/ml) was >4.76x10–10, 3.22x10–10 and 3.30x10–10, respectively (Table 8). The oxytetracycline resistant *V. campbellii* M110 yielded two bands with molecular weight >10 kb (Figure 1).

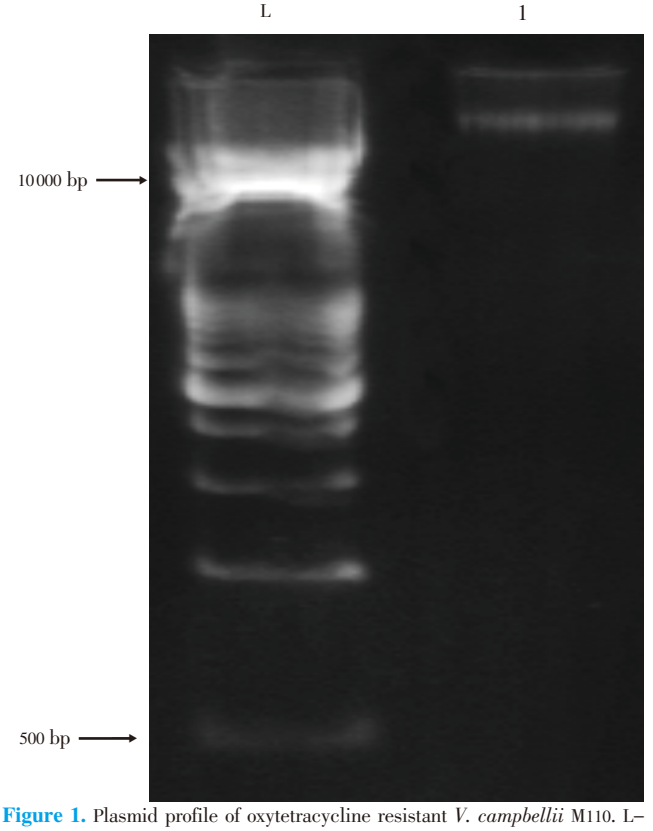

Figure 1. Plasmid profile of oxytetracycline resistant *V. campbellii* M110. L–500 bp to 10 kb, 1–plasmid DNA.

Table 8
Frequency of mutants among chloramphenicol sensitive *V. hollisae* and *V. campbellii* of *bhery*.

Bacterial strain	Source	Antibiotyping	Mutation frequency
V. hollisae–M76	Outlet sediment	AS, BS, CS, ER, FS, GS, LR, NR, OR, SS, TS, VI	$>4.76\times10^{-10}$
V. hollisae–M78	Outlet sediment	AS, BS, CS, ER, FS, GS, LR,NR, OR, SS, TS, VI	3.22×10^{-10}
V. campbellii–M110	Inlet sediment	AS, BS, CS, ER,FS, GS, LR, NI, OR, SI, TS, VR	3.30×10^{-10}

A: amoxyclav (30 μg); B: gatifloxacin (5 μg); C: chloramphenicol (30 μg); E: erythromycin (15 μg); F: ciprofloxacin (5 μg); G: gentamicin (10 μg); L: clindamycin (2 μg); N: nitrofurantoin (300 μg); O: oxytetracycline (30μg); S: sulfafurazole (300 μg); T: co–trimoxazole (25 μg); V: vancomycin (30 μg). R: resistant; I: intermediate; S: sensitive.

4. Discussion

PVCs to a maximum of 10^2/mL of pond water are recommended for shrimp culture[15]. As shown in Table 1 of the present study, the PVC range of pond water was found to be 1.00×10^2–9.30×10^3/mL, which was higher than the recommended level. The incoming water carried vibrios in the range of 5.50×10^1–1.00×10^3/mL into the *bhery*, and there appear to build up vibrios in the culture system with days of culture, as there was about 9 fold increase in vibrios. The levels of PVC were observed to be moderately higher in outlet water and ranged between 4.15×10^2 and 4.15×10^3/mL. Nevertheless, the levels of PVC were observed to be lower than the earlier studies made in other shrimp culture systems[16,17]. In an earlier study in West Bengal, PVCs in the range of $<1.00\times10^2$–4.00×10^2/mL pond water in low saline shrimp ponds, $<1.00\times10^2$–1.00×10^5/mL of pond water in medium saline ponds and 8.00×10^3–2.00×10^5/mL in high saline ponds were observed. While the range of PVC in modified extensive shrimp ponds was 10^2–10^5/mL pond water[5]. Sediment is a mixture of living organisms and dead materials in a medium of sand, clay or silt or a combination of them, which functions as a buffer and serves as a nutrient source. The PVC of pond sediment was in the range of 1.00×10^2–4.90×10^3 g. While in inlet (2.00×10^2–4.20×10^4 g) and in outlet (3.00×10^2–6.85×10^3 g) the levels of PVC were observed to be higher than the pond sediment. Significant differences existed in the levels of PVCs ($P<0.03$), sucrose negative vibrios ($P<0.02$) and sucrose positive vibrios ($P<0.03$) among the sources. The differences between the sediment PVCs of pond and outlet, and inlet and pond were significant ($P<0.05$). The differences between the sucrose negative vibrios of pond water and outlet water, and inlet water and outlet water were significant ($P<0.05$). In inlet water, the correlations between PVCs and sucrose negative vibrios, and sucrose positive vibrios were positive and significant ($P<0.05$). In pond water, there was a significant positive correlation between PVCs and sucrose positive vibrios ($P<0.05$); while in outlet water the positive correlations between PVCs and sucrose negative vibrios, and sucrose positive vibrios ($P<0.05$) were significant. The differences between the sucrose positive *Vibrio* levels of pond sediment and outlet sediment, and inlet sediment and pond sediment were significant ($P<0.05$). In inlet, outlet and pond sediment the correlations between PVCs and sucrose negative vibrios, and sucrose positive vibrios were positive and significant ($P<0.05$). The results of this study are in close agreement with the earlier observation as the pond sediment provided more nutrients for bacterial growth in comparison to overlying waters[5,16,17]. Moriarty recorded that the shrimp gut flora was often dominated by *Vibrio* species that were sucrose negative (green on TCBS agar), and these faecal flora would have contributed substantially to the sucrose negative vibrios of the pond water and sediment[8]. *Vibrio* spp. constitute the major bacterial pathogens in cultivated marine penaeid shrimp[18–20]. A total of 157 sucrose negative bacterial isolates were isolated, identified and used as biomarkers to understand the influence of *bhery* on the ecology of vibrios and antibiotic resistance. Thirteen different species of sucrose negative vibrios were observed in water and sediment samples of *bhery*, which are in conformity with earlier studies[18–20].

As shown in Table 3, sucrose negative vibrios and non–vibrios of the *bhery* exhibited varying degrees of resistance to 12 tested antibiotics. The results of the present study are in agreement with earlier studies recorded in shrimp grow–out ponds[1,19–21]. Oxytetra–cycline is one of the commonly used antibiotics in shrimp farms. The major problem of antibiotic is that bacterial pathogens can easily develop plasmid mediated resistance to it and may also enhance the frequency of new antibiotic resistant bacteria in culture system[21]. There have also been reports that increasing resistance to oxytetracycline, resistant strains can occur in an environment that had not been using the antimicrobials[22]. Bacterial resistance to chloramphenicol, oxytetracycline and co–trimoxazole is associated with plasmids. It is suggested that the plasmids carried by fish/shrimp pathogen may potentially serve as a reservoir for resistance to other antimicrobials[23]. A major concern of antibiotic usages is the acquisition of MAR[23–25]. The MAR, *i.e.*, resistance to at least two antibiotics, was noticed among 42.86% of the isolates screened, *i.e.*, 43.85% among sucrose negative vibrios and 41.86% among sucrose negative non–vibrios. The results of MAR are in accordance with Abraham and Sasmal[19]. Although no antibiotic was used in the *bhery*, the observation of MAR to the tune of 44% among the sucrose negative vibrios and non–vibrios is a cause of concern. Source–wise MAR as shown in Table 5 revealed maximum MAR in inlet sediment (53.84%) followed by outlet sediment (51.42%). The prevalence of virulent shrimp pathogen *V. harveyi* with high MAR in *bhery* is also a cause for concern as they are reported to cause major disease outbreaks in hatcheries and grow–out system[26]. The present study recorded MAR index in the range of 0–0.41. In contrast, Manjusha *et al*. observed antibiotic resistance index in the range of 0.02–0.05 among vibrios of Kerala brackishwater shrimp farms[27]. The difference in the MAR among the sucrose negative vibrios and non–vibrios was insignificant ($P>0.05$), indicating that they share a common resistance pattern.

The sucrose negative vibrios and non-vibrios of the *bhery* had 23 and 18 resistance profiles, respectively. The most common resistance profile among the sucrose negative vibrios was LV followed by LS, ST, AL, LNO, AS, ALV, AGLV, EFST, and ELNO. An earlier study indicated that MAR bacteria are highly virulent than antibiotic sensitive bacteria and when multiple resistances occurred, the single most commonly involved agent was oxytetracycline[25]. The mutation frequency of *V. hollisae* and *V. campbellii* strains to chloramphenicol (25 μg/mL) was ranged from $>4.76\times10^{-10}$ to 3.30×10^{-10}, which is well within the normal level. Generally, in bacteria spontaneous mutation occurs at a rate of 10^{-9}–10^{-10}, which enable microbes to exchange nucleotides and lead increasing mutation[28]. The frequency of mutation among the vibrios of West Bengal was lower than that of Tamil Nadu, India[26], where recorded mutation frequency in the range of 2.59×10^{-9}–1.82×10^{-8} in chloramphenicol resistant *V. harveyi* isolated from *Fenneropenaeus indicus* larvae. The MAR index was, in general, higher in inlet water and sediment samples and MAR observed in biomarker strains of pond water and pond sediment (40%), was comparable to those of inlet, thus confirming the fact that incoming water was the major source of antibiotic resistant bacteria. It seems from the results of the present study that the shrimp culture in *bhery* does not favour the proliferation and spread of antibiotic resistant bacteria. Furthermore, no finfish or shellfish diseases or abnormalities were observed during the culture period, thus indicating that the *bhery* ecosystem is an ideal ecosystem for sustainable aquaculture.

The oxytetracycline resistant *V. campbellii* M110 yielded two bands with molecular weight >10 kb, indicating that it harboured resistance determinant plasmids (Figure 1). Manjusha and Sarita in their studies recovered plasmids of size from 5.98 to 19.36 kb in vibrios isolated from crustaceans and mollusc of Kerala, India[23]. In another plasmid profiling study by Devi *et al.*[29], *Vibrio parahaemolyticus* isolates were found to have seven plasmids of size 0.75, 1.2, 6 and 8 kb and 3 plasmids above 10 kb in shrimp farms along the southwest of India. The results of this study, more or less, corroborate the earlier studies[23,29]. The isolation of R-plasmid carrying *V. campbellii* from the *bhery* ecosystem in West Bengal suggested the potential threat to humans and aquatic animals. From these results, it can be inferred that such bacteria carrying antibiotic resistant determinants may be the source of spreading antibiotic resistance to other environmental and pathogenic bacteria which share the same aquatic environment. Further study on the antibiotic resistance profile and plasmid ecology of environmental and *bhery* isolates of *Vibrio* spp. will be of special importance to understand the genetic exchanges among Gram-negative bacteria in aquatic environment.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

The occurrence of antibiotic resistant bacteria in shrimp farming system is becoming a serious problem worldwide including India. There has been a simultaneous practice of traditional shrimp farming along with external input based farming (modified extensive, semi-intensive *etc.*) in West Bengal and other maritime states of India. It is of great concern that antibiotic resistant bacteria, emerging out of input based culture system, are being detected in those farming areas where antibiotics have never been used.

Research frontiers

Microbial ecology of antibiotic resistant vibrios in traditional shrimp farming system of West Bengal, India has been studied. This is important because such vibrios have been detected in those farming areas where antibiotics have never been used.

Related reports

Reports regarding the antibiotic resistant bacteria in shrimp farming system are substantial. However, microbial ecology of bacteria, especially antibiotic resistant bacteria from traditional farming system where antibiotics are not used, is limited.

Innovations and breakthroughs

The finding that antibiotic resistant vibrios were associated with the traditional shrimp farming system is of importance from risk assessment point of view.

Applications

Through quantification of bacterial flora and plasmid profiling of antibiotic resistant bacteria, it has become clear that traditional shrimp aquaculture is facing a threat from the use of antibiotics in the adjacent areas. This study will help to assess risk associated with disease causing bacteria particularly vibrios.

Peer review

This is a good study in which the authors found presence of multiple antibiotic resistant bacteria in traditional shrimp farming system. The outcome is interesting because antibiotic resistant bacteria, emerging out of input based culture system, was detected in those farming areas where antibiotics have never been used.

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