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Frequency and Expression of Antibiotic Resistance in Luminous *Vibrio harveyi*

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In-vitro activities of chloramphenicol, ciprofloxacin, nalidixic acid and oxytetra-cycline were evaluated against penaeid shrimp larval pathogen, luminous *Vibrio harveyi*. The frequency at which three strains of luminous *V. harveyi* strains can mutate to develop resistance to these antibiotics was also estimated. Resistant mutants developed at lower frequencies to chloramphenicol ($<9.43 \times 10^{-10}$) and ciprofloxacin ($<1.14 \times 10^{-10} - 1.61 \times 10^{-9}$) than to nalidixic acid ($6.61 \times 10^{-5} - 8.87 \times 10^{-5}$) and oxytetracycline ($6.82 \times 10^{-6} - 4.74 \times 10^{-4}$) at 10 times the respective minimal inhibitory concentration (MIC). Low concentration of antibiotics (5 x MIC and 2 x MIC), however, induced more of mutant strains. Although ciprofloxacin was effective in terms of MIC and induction of low level of mutants, chloramphenicol was more effective in its ability to kill both resistant and sensitive strains of luminous *V. harveyi*.

Key words: ***Vibrio harveyi*, antibiotic resistance, mutation frequency, shrimp hatchery.**

In recent years, *Vibrio harveyi* has become recognized globally as a devastating pathogen of penaeid shrimp larvae (Lavilla-Pitogo *et al.*, 1990; Baticados *et al.*, 1990; Karunasagar *et al.*, 1994; Abraham *et al.*, 2001). Highly virulent strains of *V. harveyi* result up to 100% mortality from bath inocula containing as few as 10^2-10^3 cells / ml (Lavilla-Pitogo *et al.*, 1990; Karunasagar *et al.*, 1994). The use of drugs in shrimp hatcheries has, more often than not, been the practice in almost all shrimp producing countries of the world. A large array of drugs including chloramphenicol, ciprofloxacin, co-trimoxazole, enrofloxacin, erythromycin, furazolidone, rifambicin, oxolinic acid, oxytetracycline, prefuran, rifambicin, streptomycin, rifambicin and others have been used in shrimp hatcheries to combat luminous vibriosis (Baticados & Paclibare, 1992; Karunasagar *et al.*, 1994). The effectiveness of potential antibiotics has been hampered by the development of resistance in *V. harveyi*, which seems to be occurring with increasing frequency (Baticados *et al.*, 1990; Karunasagar *et al.*, 1994; Abraham *et al.*, 2001). Reports on the frequencies at which the shrimp larval pathogen, *V. harveyi* mutated to develop resistance to potent antibiotics are scarce and, therefore, determined in this study besides the investigation on killing efficiency of these antibiotics in seawater microcosms.

Materials and Methods

Three strains of luminous *Vibrio harveyi* (strains A_3, B_5 and A_{34}) isolated from hatchery produced shrimp larvae (Abraham *et al.*, 1999) in Tuticorin, Tamil Nadu, India

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were selected for this study. All bacterial strains were maintained on seawater complex (SWC) agar slants at 28°C (Nealson, 1978). Overnight grown cultures used in the experiments were prepared by inoculating young cells into SWC medium and incubating at 30±2°C. Four antibiotics, viz., chloramphenicol, ciprofloxacin, nalidixic acid and oxytetracycline (Himedia, Mumbai) were selected for the *in-vitro* and mutation frequency experiments. Chloramphenicol, ciprofloxacin and nalidixic acid were first dissolved, respectively in 1:10 ethanol, 0.01 N hydrochloric acid and 0.1 M - sodium hydroxide. Further dilutions to a required concentration were made in sterile distilled water. Oxytetracycline was dissolved in sterile distilled water. Fresh antibiotic solutions were prepared for all experiments.

Minimal inhibitory concentrations (MIC) were determined by agar dilution method on Mueller-Hinton agar supplemented with 1.0% (w/v) sodium chloride (MHA) as described by Mohney *et al.* (1992). The MIC was determined as the minimum concentration (mg/ml) showing no growth at 24 h. Mutation frequency was estimated by the method of Barnes *et al.* (1991). Bacterial cells, grown on SWC agar without glycerol for 20 h, were harvested in half strength-aged seawater (final salinity: 17.5 ppt) and washed twice by centrifugation at 6,000 rpm for 10 min. The deposits were then resuspended in 5 ml half strength aged seawater to a concentration of 10^9-10^{10} cells/ml. Aliquots (0.1 ml) of cell suspensions from undiluted, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} dilutions were then spread onto MHA containing antibiotics at 2, 5 and 10 times the MIC in duplicate. Simultaneously, 0.1 ml each of serially diluted (10^{-5}, 10^{-6} and 10^{-7}) cell suspensions was spread onto antibiotic-free medium. The seeded agar plates were incubated at 30±2°C for 5-7 days and the numbers of growing colonies were counted. Mutation frequency was calculated as the number of colonies on MHA with antibiotic divided by the number of colonies on antibiotic-free MHA.

The killing effect of antibiotics such as chloramphenicol, ciprofloxacin and oxytetracycline at two different concentrations above the MIC in sterile aged seawater was investigated by inoculating bacterial cells into the flasks containing antibiotic incorporated seawater at a level of about 10^7 cells/ml. Aliquots (0.1 ml) of sample were drawn aseptically from the flasks immediately after inoculation and at 2, 6 and 10 h of post-inoculation. The number of viable cells was determined on spread plates with SWC agar and the percentage survival calculated. All the experiments were done in duplicate and the average results are presented.

Results and Discussion

As seen in Table 1, *Vibrio harveyi* strains A_3 and A_{34} were resistant to chloramphenicol, showing MIC values 100 and 75 µg/ml, respectively. The strain B_5 was resistant (MIC 75 µg/ml) to oxytetracycline. The MIC values for ciprofloxacin were in the range of 0.39- 0.78 µg/ml. It has been estimated that Indian shrimp hatcheries typically use, on an average, 1.00 kg of furazolidone or 0.30–0.50 kg of oxytetracycline/ chloramphenicol or 0.40-0.50 kg of other antibiotics (ciprofloxacin, erythromycin, gentamycin, oxolinic acid, co-

trimoxazole, etc.) to produce one million post-larvae. The results on high MIC with luminous strains suggest that numerous other bacterial strains with high degree of antibiotic resistance may be present in shrimp hatchery. Antibiotic resistance among shrimp pathogens is a well-known phenomenon and is amply documented in several earlier studies (Baticados *et al.*, 1990; Mohney *et al.*, 1992; Karunasagar *et al.*, 1994; Abraham *et al.*, 2001).

Mutation frequencies give some idea of the rate at which resistance to an antibiotic is likely to develop during therapy. As seen in Table 1, mutation frequencies for chloramphenicol-sensitive *V. harveyi* B_5 to chloramphenicol and ciprofloxacin were very low at 5 and 10 times the respective MIC than at 2 times the MIC. The results corroborate with the observations of Barnes et al. (1991) recorded on *Aeromonas salmonicida*. The present results suggest that resistance may most likely develop in bacteria where these antibiotics are abused. This perhaps provides an explanation for the prevalence of oxytetracycline and chloramphenicol-resistant *V. harveyi* strains in shrimp hatcheries. The data also suggest that in antibiotic-sensitive luminous strain resistance may less likely develop clinically to ciprofloxacin than for chloramphenicol. Mutation frequencies for luminous *V. harveyi* strains to ciprofloxacin recorded in this study ($<1.14\times10^{-10}$ - $<1.61\times10^{-9}$) are in agreement with Nakano *et al.* (1989) and Barnes *et al.* (1991) observed on *V. anguillaram* and *A. salmonicida*, respectively.

Table 1. Mutation frequencies of *Vibrio harveyi* strains

Bacterial strains/ antibiotics	MIC (µg/ml)	Mutation frequency 5 x MIC	10 x MIC
Vibrio harveyi A_3			
Chloramphenicol	100	NT	NT
Ciprofloxacin	0.78	$<5.68\times10^{-10}$	$<1.14\times10^{-10}$
Nalidixic Acid	6.25	2.01×10^{-2}	7.76×10^{-5}
Oxytetracycline	6.25	1.28×10^{-3}	6.82×10^{-6}
Vibrio harveyi B_5			
Chloramphenicol	3.13	9.43×10^{-10}	$<9.43\times10^{-10}$
Ciprofloxacin	0.78	1.89×10^{-9}	$<9.43\times10^{-10}$
Nalidixic Acid	6.25	2.15×10^{-4}	8.87×10^{-5}
Oxytetracycline	75.00	NT	NT
Vibrio harveyi A_{34}			
Chloramphenicol	75.00	NT	NT
Ciprofloxacin	0.39	1.61×10^{-9}	$<1.61\times10^{-9}$
Nalidixic Acid	6.25	4.74×10^{-4}	6.61×10^{-5}
Oxytetracycline	6.25	1.62×10^{-3}	4.74×10^{-4}

Mutation frequencies for three strains of *V. harveyi* at 2 x MIC of chloramphenicol and ciprofloxacin were in the range of 2.87×10^{-7} to 1.23×10^{-4} and 1.14×10^{-9} to 2.92×10^{-7}, respectively.

MIC: Minimal inhibitory concentration

NT: Not Tested

Mutation frequencies to oxytetracycline varied markedly among the oxytetracycline-sensitive *V. harveyi* strains. Low concentration of oxytetracycline (5xMIC) induced more of mutant strains than at high concentration (10xMIC). The rate of mutant formation among *V. harveyi* strains was, however, high in the presence of oxytetracycline than for chloramphenicol and ciprofloxacin (Table 1). Frequencies of oxytetracycline-resistant mutants were also reportedly low ($<6.10\times10^{-10}$ - 6.82×10^{-7}) in *A. salmonicida* at 5 and 10 times the MIC (Barnes *et al.*, 1991). Unlike ciprofloxacin, mutation frequencies to nalidixic acid were observed to be high and varied among the nalidixic acid-sensitive *V. harveyi* strains (Table 1). This high rate of mutation frequencies for nalidixic acid and

Table 2. Survival of *Vibrio harveyi* strains in antibiotic incorporated seawater.

Bacterial strains/ antibiotics	Test concentration (µg/ml)	Percentage survival in 0 h	2 h	6 h	10 h
Vibrio harveyi A_{3a}					
Chloramphenicol	150	100	21.03	12.17	5.34
	300	100	18.74	11.02	3.27
Ciprofloxacin	1	100	56.78	49.73	35.43
	3	100	43.47	38.31	29.17
Oxytetracycline	10	100	69.71	58.73	49.15
	30	100	40.13	25.31	17.63
V. harveyi B_{5b}					
Chloramphenicol	10	100	4.56	3.63	2.72
	30	100	3.37	2.17	0.53
Ciprofloxacin	1	100	54.64	31.12	11.71
	3	100	51.65	20.00	5.30
Oxytetracycline	150	100	50.11	31.11	18.01
	300	100	26.67	21.01	11.64
V. harveyi A_{34c}					
Chloramphenicol	150	100	19.96	6.67	1.11
	300	100	16.64	3.06	0.85
Ciprofloxacin	1	100	57.79	52.33	34.67
	3	100	47.33	41.67	26.00
Oxytetracycline	10	100	72.67	67.67	55.01
	30	100	33.37	21.67	15.56

a, b and c refer to initial bacterial counts (100%): $2.53\pm1.03\times10^7$/ml, $3.88\pm2.08\times10^7$/ml and $2.89\pm0.95\times10^7$/ml, respectively.

oxytetracycline indicated the danger of using these antibiotics in shrimp aquaculture. Frequent use of these antibiotics may enhance the frequency of new oxytetracycline and/or nalidixic acid resistant isolates in the system as observed by Williams *et al.* (1992). It appears from the results of mutation frequency experiments that ciprofloxacin is a potentially useful therapeutic agent to combat luminous vibriosis in shrimp hatcheries.

Prior to recommendation of antibiotics for use in shrimp aquaculture, it is of interest as a first step to explore the killing rates. As shown in Table 2, *V. harveyi* strains were not eliminated by the addition of chloramphenicol, ciprofloxacin and oxytetracycline at concentrations above the MIC in seawater microcosms. Chloramphenicol was bactericidal to >95 % of chloramphenicol-sensitive *V. harveyi* B_5 within first 2 h of exposure and bacteriostatic thereafter. While in chloramphenicol-resistant *V. harveyi* strains, the observed bactericidal activity was in the range of 79-85% in first 2 h. Chloramphenicol is known for its wide spectrum of antimicrobial activity, but the results revealed that it exerted marked prophylactic and therapeutic effects only against sensitive strains. In contrast, ciprofloxacin was bacteriostatic to *V. harveyi* at 1 and 3 mg/ml levels in seawater microcosms. Although ciprofloxacin was active in terms of MIC and induced very low level of

mutants than chloramphenicol (Table 1) it exerted only bacteriostatic action against *V. harveyi* in seawater microcosms (Table 2) which, in turn, limit the use this potent antibiotic to control luminous vibriosis. Lewin & Hastings (1990) also observed that ciprofloxacin was not bactericidal against non-dividing *A. salmonicida* at 9mg/ml level in phosphate buffered saline. However, they found that ciprofloxacin was able to kill oxolinic acid-sensitive and resistant strains of *A. salmonicida* at concentrations above the MIC within 3 h under conditions in which bacteria were able to divide. Therefore, it would appear from the results of these investigations that ciprofloxacin may not be active against non-dividing *V. harveyi*. Oxytetracycline also exhibited bacteriostatic action against *V. harveyi* in seawater microcosms (Table 2). Karunasagar *et al.* (1994) demonstrated that luminous bacteria could survive in the presence of chloramphenicol, erythromycin, neomycin, oxytetracycline, furazolidone and nifurprinol in seawater microcosms even at 1,000 mg/ml level. The results presented here and also that of Baticados et al. (1990) showed that the currently used antibiotics in shrimp aquaculture are ineffective in controlling the *V. harveyi*. The limitation of chemical treatment of luminous vibriosis among the shrimp larvae restricts the use of this method of control.

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