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Biofilm formation by *Vibrio harveyi* on surfaces

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Abstract

The role of biofilm in the survival and persistence of the bacterial shrimp pathogen *Vibrio harveyi* and its possible role in perpetuating infection in shrimp hatcheries was studied. *Vibrio harveyi* formed biofilms on all three substrates tested: cement slab, high density polyethylene (HDPE) plastic and steel coupons. Cell density was highest on the plastic surface followed by the cement slab and the steel surface. Biofilm on the three surfaces also exhibited differential sensitivity to the sanitiser chlorine, maximum resistance being found on the concrete slab followed by plastic and steel coupons. Planktonic cells were sensitive to short exposure to low levels of chlorine. Biofilm formation occurred even in the presence of the antibiotics chloramphenicol and tetracycline, both added to the medium at 50 ppm.

Keywords: Biofilm; *Vibrio harveyi*; Substrate; Sanitiser; Chlorine, Antibiotic

1. Introduction

Mortalities in larvae of the shrimp *Penaeus monodon* due to infection by luminous *Vibrio harveyi* have been reported from a number of countries (Lightner, 1983; Sunaryanto and Mariam, 1986; Tansutapanit and Ruangpan, 1987; Lightner, 1988; Lavilla-Pitogo et al., 1990; Baticados et al., 1991; Karunasagar et al., 1994). Since this organism is a part of the autochthonous flora of the marine environment (Baumann and Baumann, 1981), the source of infection is suspected to be near shore water (Lightner et al., 1992). To combat this problem, most shrimp hatcheries use water treatment measures such as sand filters and chlorination. Nevertheless, *V. harveyi* can be found in larval rearing tanks in considerable numbers (Karunasagar et al., 1994). To provide an

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explanation for survival and persistence of *V. harveyi* in larval tanks even after addition of antibiotics, we considered the possibility that this organism formed biofilms on surfaces. In contrast to planktonic bacteria, biofilm bacteria have been reported to be protected against antibacterial agents (Costerton et al., 1987). Against this background, we investigated the resistance of *V. harveyi* biofilm to water sanitisers.

2. Materials and methods

A culture of *V. harveyi* isolated from moribund shrimp larvae was used. The culture was identified by biochemical tests indicated in Table 1. The virulence of the culture to *P. monodon* post larvae (PL 18-20) was tested as described earlier (Karunasagar et al., 1994). The culture gave an LD_{50} of 1.8×10^4 cells. Biofilm formation was tested on concrete slabs (58 cm^2), stainless steel and plastic (high density polyethylene, HDPE) coupons of about 60 cm^2. Concrete slabs were scrubbed with a brush and washed well with distilled water before use. Stainless steel coupons were cleaned with acetone to remove grease, etched by submerging in 5 N HCl for 15 min, cleaned in detergent solution and finally rinsed in type I reagent grade water (Ren and Frank, 1993). The HDPE coupons were cleaned in detergent solution and rinsed in type I reagent grade water. Biofilm cells were grown as described by Frank and Koffi (1990) using diluted tryptic soya broth (TSB) prepared as follows: 2 g TSB dehydrated powder (Hi Media, Bombay) was dissolved in 10 ml distilled water and sterilised by autoclaving at 121°C

Table 1
Biochemical characteristics of *V. harveyi* isolated from infected larvae

Characteristic	Reaction as per West and Colwell (1984)	Reaction of the isolate used
Cytochrome oxidase	+	+
Nitrate reduction	+	+
O/129 sensitivity (150 μg)	+	+
Swarming	–	–
Luminescence	V	+
Thornley's arginine dihydrolase	–	–
Lysine decarboxylase	+	+
Ornithine decarboxylase	+	+
Growth at 0% NaCl	–	–
Growth at 3% NaCl	+	+
Growth at 8% NaCl	+	+
Growth at 10% NaCl	V	+
Voges–Proskauer reaction	V	–
Gas from glucose	–	–
Fermentation to acid:		
L-arabinose	–	–
m-inositol	–	–
D-mannose	+	+
Sucrose	V	–

V, variable.

for 20 min. A 1 ml aliquot of this medium was added to 99 ml pre-sterilised seawater. This resulted in a TSB level of 8% of its usual concentration in seawater. The substrates for biofilm formation were autoclaved individually in 250 ml glass beakers and 100 ml diluted TSB were added to each beaker. The medium was inoculated with 2 ml of a 24 h culture of *V. harveyi* in TSB containing 1% sodium chloride (TSBS). After 2 days at room temperature (28 ± 2°C), the coupons were aseptically removed, washed in sterile phosphate buffered saline (PBS) to remove unattached cells and placed in beakers of fresh sterile medium. The procedure was repeated for five cycles during 10 days of growth. Biofilm formation was also attempted in the presence of the antibiotics tetracycline and chloramphenicol. The only difference to the procedure described above was the addition of 50 ppm tetracycline and chloramphenicol to the low nutrient medium.

To enumerate biofilm cells, the coupons were washed in sterile PBS and the cells removed by rubbing with sterile cotton swabs. The swabs were transferred to 100 ml PBS, shaken vigorously and serial ten-fold dilutions were plated on tryptic soy agar (Hi Media, Bombay) containing 1% sodium chloride (TSAS). To test the sensitivity of biofilm cells to the sanitiser, the coupons were rinsed in PBS and dipped in 20, 100 and 200 ppm hypochlorite solution for 10 min. Coupons were then transferred to neutralising solution (0.01 M sodium thiosulphate) for 30 s, rinsed with PBS and cells enumerated after swabbing as described above. The control coupon was dipped in sterile tap water, washed and processed as the treated coupons.

Sensitivity of planktonic cells to the sanitiser was tested as follows: a 24 h culture of *V. harveyi* in TSBS was centrifuged at $8000 \times g$ for 10 min and the cell pellet suspended in the same volume of filter sterilised seawater. One millilitre of this was then added to 100 ml of sterile seawater and enumerated on TSAS. To this suspension, hypochlorite was added to give a final concentration of 20 ppm available Cl_2 and at 5 and 10 min, aliquots were taken, diluted and plated as described above.

3. Results and discussion

As shown in Table 2, *V. harveyi* formed biofilm on all three surfaces tested. Cell density was highest on HDPE (5.3×10^7 colony forming units (CFU) cm^{-2}), followed by concrete slab (8.5×10^6 CFU cm^{-2}) and steel coupon (2.4×10^6 CFU cm^{-2}).

Table 2
Biofilm formation by *V. harveyi* on different surfaces and their sensitivity to chlorine (CFU cm^{-2})

Type of substrate	Control	Levels of chlorine and exposure time		
		20 ppm 10 min	100 ppm 10 min	200 ppm 10 min
Cement slab	8.49×10^6	6.62×10^5	5.67×10^4	4.36×10^3
Plastic (HDPE)	5.34×10^7	2.44×10^5	3.40×10^3	–
Steel coupon	2.44×10^6	3.88×10^3	–	–

Table 3
Biofilm formation by *V. harveyi* (CFU cm^{-2}) in the presence of the antibiotics tetracycline and chloramphenicol (both added at 50 ppm)

Type of substrate	Control	Tetracycline	Chloramphenicol
Plastic	5.34×10^7	5.59×10^7	3.08×10^6
Cement slab	1.23×10^7	1.17×10^7	1.14×10^7
Steel coupon	2.44×10^6	7.18×10^6	1.08×10^7

Interestingly, biofilm formation took place even in the presence of the antibiotics tetracycline and chloramphenicol (Table 3). Using the disc diffusion method, it was found that *V. harveyi* was sensitive to chloramphenicol (30 μg disc strength) and tetracycline (30 μg), zones of inhibition measuring 22 mm and 24 mm, respectively. Nevertheless, biofilm formation took place in the presence of these antibiotics. These results have implications for shrimp hatcheries which use antibiotics as prophylactics in larval tanks. The results suggest that *V. harveyi* entering a hatchery via water, spawners or eggs can survive by forming a biofilm on the surface of the tanks. These biofilm bacteria could be a source of infection for the larvae.

Results in Table 2 show that biofilm cells of *V. harveyi* are highly resistant to the effect of sanitiser. Planktonic *V. harveyi* cells suspended in seawater to a level of 2.1×10^6 ml^{-1} were completely killed by exposure to hypochlorite solution at available chlorine of 20 ppm for 5 min. However, biofilm *V. harveyi* cells, particularly on concrete slabs and HDPE showed only a slight reduction in numbers after 10 min exposure to 20 ppm chlorine. A similar exposure to 100 ppm chlorine completely killed biofilm cells on steel coupons whereas biofilm cells on HDPE were partially inactivated. The extent of reduction in counts was 2 log units for concrete slabs and 4 log units for HDPE slabs. A 10 min exposure to 200 ppm chlorine completely eliminated biofilm cells on HDPE coupons but concrete slabs still had 4.4×10^3 *V. harveyi* cm^{-2} even after exposure to such a high level of chlorine. These results suggest that biofilm cells of *V. harveyi* are highly resistant to treatment by sanitisers and the extent of resistance depends on the surface. Biofilms on steel coupons were most sensitive, followed by HDPE. These results are of importance to the shrimp hatcheries. Biofilm formation can take place on surfaces of water storage tanks, polythene water pipes and on surfaces of larval tanks. Such biofilm bacteria are not easily eliminated by sanitiser treatment and could be a source of infection for the larvae. These results explain our earlier report (Karunasagar et al., 1994) of persistence of antibiotic resistant *V. harveyi* in larval tanks of a hatchery with an extensive water treatment system and using antibiotics as prophylactics. The results emphasise the need for physical removal of biofilm on tank surfaces and periodical drying of tanks to reduce the chances of infection by organisms such as *V. harveyi*.

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