



ELSEVIER

Aquaculture 191 (2000) 133–144

Aquaculture

www.elsevier.nl/locate/aqua-online

Experimental infection models for shrimp vibriosis studies: a review

Denis Saulnier ^{a,*}, Phillipe Haffner ^a, Cyrille Goarant ^b,
Peva Levy ^a, Dominique Ansquer ^a

^a *Laboratoire d'Aquaculture Tropicale, Centre Océanologique du Pacifique, IFREMER, BP 7004, 98719 Taravao, Tahiti, French Polynesia*

^b *Laboratoire IFREMER, BP2059, Nouméa, New Caledonia*

Received 1 March 2000; accepted 9 May 2000

Abstract

Vibrio species have become a major source of concern for shrimp culture because of their close association with low survival rates in hatcheries or growout ponds. New shrimp pathogens belonging to the *Vibrio* genus have been described although their virulence is not yet fully understood. Indeed, they may act as opportunistic agents in secondary infections or be true pathogens. This review presents the usefulness of infection models with vibriosis pathogens for pathogenicity experiments, testing of curative or prophylactic treatments and the study of host-factors influencing bacterial virulence. Furthermore, some guidelines for experimental trials are given to evaluate the in vivo virulence of *Vibrio* isolates. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Shrimp vibriosis; Infectious pathogens; Infection models

1. Introduction

The intensification of the penaeid shrimp culture industry and the transfer of aquatic organisms worldwide have been accompanied over the last two decades by an increased incidence of infectious pathogens. In this context, bacterial diseases due mainly to *Vibrio* species are often associated with low survival rates in hatchery or growout conditions. Larval mortalities associated with the presence of *V. harveyi* have been

* Corresponding author.

E-mail address: Denis.Saulnier@ifremer.fr (D. Saulnier).

reported in *Penaeus monodon* and *P. vannamei* in Indonesia (Sunaryanto and Mariam, 1986), Thailand (Jiravanichpaisal et al., 1994), India (Karunasagar et al., 1994), Philippines (Baticados et al., 1990; Lavilla-Pitogo et al., 1990), Australia (Pizzutto and Hirst, 1995), Taiwan (Song and Lee, 1993; Liu et al., 1996a) and Ecuador (Robertson et al., 1998). Disease outbreaks attributed to other *Vibrio* species such as *V. alginolyticus*, *V. damsela*, *V. parahaemolyticus*, *V. vulnificus* and *V. penaeicida* have been observed in nursery or growout ponds of *P. vannamei*, *P. monodon*, *P. japonicus* and *P. stylirostris* in Ecuador (Lightner, 1992), Malaysia (Anderson et al., 1988), Taiwan (Song et al., 1993; Lee et al., 1996), Philippines (Alapide-Tendencia and Dureza, 1997), Japan (Takahashi et al., 1985a; de la Peña et al., 1993) and New Caledonia (Costa et al., 1998; Mermoud et al., 1998).

Disease signs range from localized cuticular lesions, oral and enteric infections to septicemia (Lightner, 1996). The natural abundance of *Vibriosp.*, their ubiquity, multiplication rates and ability to adapt to environmental changes in shrimp culture ecosystems increase these preoccupations. Nevertheless, their precise aetiological role remains uncertain and, as a consequence, they have been described in a wide range of shrimp disease either as opportunistic agents or true pathogens, and sometimes alternately.

The usefulness of in vivo experimental infection models with vibriosis pathogens and the methodological approach to evaluate the virulence of *Vibrio* isolates will be presented in this review.

2. Why develop pathogenicity assays with *Vibrio* spp.?

2.1. Demonstration of bacterial virulences

In order to ascertain the pathogenicity of dominant *Vibrio* isolates associated with disease outbreaks and verify Koch's Postulates, pathogenicity experiments have been conducted in numerous strains (see Table 1). In most cases, a high inoculum was needed to reproduce the disease and to reisolate the inoculated bacteria from the experimentally infected shrimp (Lightner, 1988). Moreover, pathogenic *Vibrio* isolates have also been detected in apparently healthy shrimp (Nakai et al., 1997; Vandenberghe et al., 1998) and in seawater samples from near-shore and estuary areas, where shrimp farms rearing water is pumped and from affected farms, (Lightner, 1992; Lavilla-Pitogo et al., 1990, 1998; Moriarty, 1998) as well as in sediment (de la Peña et al., 1992). These observations lead researchers to consider *Vibrio* diseases as secondary infections due to opportunistic pathogens and occurring only in immunologically compromised shrimps. Primary causes could encompass other infectious agents, nutritional deficiencies or intoxication, environmental and management practices, and induced stress.

Nevertheless, in other virulence studies (see Table 1), low number of *V. penaeicida* bacterial cells were shown to produce the disease in *P. japonicus* and *P. stylirostris* (de la Peña et al., 1993, Saulnier et al., 2000), suggesting they could act as primary

pathogens as well as some *V. harveyi* strains in *P. monodon* larvae (Lavilla-Pitogo et al., 1990; Le Groumellec et al., 1995).

2.2. Test curative and prophylactic treatments in experimental conditions

The in vitro susceptibility of particular bacterial isolates associated with shrimp disease to different antimicrobials has been studied (Baticados et al., 1990; Mohny et al., 1992, 1994). However, in vivo studies have not been conducted in shrimp to demonstrate the efficiency of such drugs in experimental conditions. Indeed, the evaluation of drug efficacy on the farms (Takahashi et al., 1985b), which is the only in vivo approach used today, could result in erroneous conclusions due to biotic or abiotic parameters such as water temperature; salinity; presence of additional pathogens; management practices; factors of host-sensitiveness to infection, such as physiological states (age, moulting) or/and genetics; and the failure of the selected route for administration (e.g. abnormal behaviour of diseased shrimp resulting in non-ingested medicated food, antibiotics, which do not reach the target infected tissues).

Empirical methods commonly used for bacterial disease management, such as partial harvesting or reduction of the biomass, use of quicklime (CaO) in ponds (Anderson et al., 1988) and increased daily water exchanges, could also be tested with a more precise idea of their impact through their use in experimental infections. In the same way, the beneficial effects of bacteria defined as probiotics and displaying antagonistic properties towards pathogenic bacteria could be evaluated in experimental infections as it has been done by Rengpipat et al. (1998).

In contrast to the situation encountered in the shrimp industry, the efficiency of probiotics or antibacterial treatments for farmed fish has been well established through controlled experimental conditions including challenges with pathogenic bacteria (Katae et al., 1979; Colorni et al., 1998; Gram et al., 1999). The determination in laboratory conditions of adequate and effective treatment for each pathogenic bacteria inducing disease outbreaks in shrimp is of importance because of the relatively small number of antibiotics legally available and the increasing number of antibiotic-resistant bacterial isolates (Karunasagar et al., 1994).

An experimental approach has been developed for a prophylactic treatment in shrimp by Itami et al. (1989), who showed that immunostimulation of juvenile *P. japonicus* with formalin-killed *Vibriosp.* provided some protection towards an intramuscular (IM) challenge by the *Vibrio* of the same species.

2.3. Evaluation of bacterial virulence according to host factors: species, age and physiological state

The species-susceptibility of shrimp to *Vibrio* infection has been studied by Hameed (1995), who demonstrated that the LD50 values of *V. campbellii*-like bacteria (see Table 1) varied among three species of shrimp (*P. monodon*, *P. indicus* and *P. semisulcatus*). Nevertheless, the bacterial suspension used for the bath challenge was high, with a final

Table 1
Results of pathogenicity experiments with *Vibrio* spp. bacteria isolated from diseased animals

<i>Vibrio</i> species, strain denomination when indicated	Shrimp species	LD50	Route of infection (and survey duration)	Reference
<i>V. alginolyticus</i> C58, C59, C53, C64A, C64B, C63B strains	<i>P. setiferus</i> , <i>P. duorarum</i> , <i>P. aztecus</i> (10 cm)	$< 10^5$ CFU/ shrimp	IM (48 h)	Lightner and Lewis, 1975
<i>V. alginolyticus</i> Val strain	<i>P. monodon</i> (13 g)	1.1×10^5 CFU/ g of body weight	IM (7 days)	Lee et al., 1996
<i>V. campbellii</i> -like ^a	<i>P. monodon</i>		Bath (24 h for N and 72 h for protozoa, Mysis and post-larva)	Hameed, 1995
	N and M	$3.5 \times 10^6 - 3.5 \times 10^7$		
	Z	CFU/ml $< 3.5 \times 10^6$ CFU/ml		
	PL	$3.5 \times 10^7 - 3.5 \times 10^8$ CFU/ml		
	<i>P. indicus</i>			
	N and M	$3.5 \times 10^7 - 3.5 \times 10^8$ CFU/ml		
	Z	$3.5 \times 10^7 - 3.5 \times 10^8$ CFU/ml		
	PL	CFU/ml $> 3.5 \times 10^9$ CFU/ml		
	<i>P. semiscalatus</i>			
	N and M	$3.5 \times 10^7 - 3.5 \times 10^8$ CFU/ml		
	Z	$3.5 \times 10^6 - 3.5 \times 10^7$ CFU/ml		
	PL	$3.5 \times 10^8 - 3.5 \times 10^9$ CFU/ml		
<i>V. harveyi</i> 770527 strain	<i>P. monodon</i> (5–6 g)	8.2×10^4 CFU/shrimp	IM (7 days)	Liu et al., 1996b
<i>V. harveyi</i> 820514 strain		8.7×10^4 CFU/shrimp		
<i>V. harveyi</i> In1 strain		7.7×10^4 CFU/shrimp		

<i>V. harveyi</i> A1 strain ^b		4.9×10^4 CFU/shrimp		
<i>V. harveyi</i>		10^6 – 10^7 CFU/shrimp		
<i>V. harveyi</i>	<i>P. monodon</i> larvae	10^2 – 10^3 CFU/ml ^c	Bath (40–48 h)	Lavilla-Pitogo et al., 1990
<i>V. harveyi</i>	<i>P. vannamei</i>	10^6 – 10^7 CFU/ml	Bath of 2 h	Robertson et al., 1998
STD3-101 strain				
<i>V. harveyi</i>	<i>P. monodon</i>	< 10^2 CFU/ml	Bath (2 days)	Le Groumellec et al., 1995
BL1 strain	larvae (zoeal)			
<i>V. harveyi</i> from larval tank	<i>P. monodon</i> PL	2.6×10^3 CFU/ml	Bath	Karunasagar et al., 1994
<i>V. harveyi</i> from seawater	<i>P. monodon</i> PL	1.5×10^5 CFU/ml		
<i>V. harveyi</i> BP04 strain ^c	<i>P. monodon</i> larvae		Bath (48 h)	Prayitno and Latchford, 1995
	Z	< 10^3 CFU/ml		
	M and PL	> 10^3 CFU/ml		
<i>V. parahaemolyticus</i>	<i>P. monodon</i> (8–12 g)	10^5 – 10^6 CFU/shrimp	IM (7 days)	Alapide-Tendencia and Dureza, 1997
<i>V. penaeicida</i> KN-1	<i>P. japonicus</i> (13–22 g)		IM	de la Peña et al., 1993
		6.3×10^2 – 6.3×10^3 CFU/shrimp		
KT-7		9×10^1 – 9×10^2 CFU/shrimp		
<i>V. penaeicida</i> AM101 strain	<i>P. stylirostris</i>	< 5 CFU/shrimp	IM (2 days)	Saulnier et al., 2000
		1.3×10^4 CFU/ml	Bath of 2 h (5 days)	

Abbreviations: N, Nauplius; Z, Protozoa, M, Mysis, PL, Post-larva, IM, Intra-muscularly.

^aIsolated from diseased larvae *P. indicus*.

^bIsolated from diseased *P. japonicus*.

^cLethal dose 50 take account of the the percentage of survival in control.

concentration of 3.5×10^6 – 3.5×10^9 CFU/ml. In another study, Le Groumellec et al. (1995) compared two strains of *V. harveyi*, named B8-2 and BL1, isolated from *P. vannamei* and *P. monodon* hatcheries in Ecuador and Thailand, respectively, during mass mortalities of *P. monodon*. Results of the crossed challenges with the two *Vibrio* strains on these two species of shrimp revealed that these strains expressed a specific virulence against their natural host. Similar results were obtained on two virulent strains belonging to *V. penaeicida*, but isolated from geographically distinct areas in two shrimp species, *P. japonicus* and *P. stylirostris* (Saulnier et al., 2000).

Studying the age-dependant susceptibility of shrimp to *Vibrio* infection, Goarant et al. (1998) showed that *P. stylirostris* post-larvae may be considered resistant to *V. penaeicida* infection; however, the animals become sensitive as soon as they reach the juvenile status, characterized by the definitive rostral formula (PL9). Similarly (see Table 1), *V. harveyi* appeared to be more virulent at zoea and mysis stages than at PLs stages (Lavilla-Pitogo et al., 1990; Prayitno and Latchford., 1995). The implication of *V. harveyi* in disease problems in growout ponds was signaled, but without pathogenicity tests (Lavilla-Pitogo et al., 1998) or with a high concentration of bacteria in an IM challenge (Alvarez et al., 1998). In another study, Le Moullac et al. (1997) demonstrated that LD50 of *V. penaeicida* in *P. stylirostris* juveniles was lower in premoult stage than in intermoult stage.

2.4. Study of the host-defense mechanisms

Huang et al. (1981) evaluated the protective and immunological effects of an injection of formalin killed *V. anguillarum* in *Macrobrachium rosenbergii*. Survival rates obtained after pathogenicity experiments with 10^7 live *V. anguillarum*/animal and expression levels of serum agglutinins were measured. Unfortunately, immunostimulation was shown to have no effect on these criteria.

3. Development of pathogenicity experiments

3.1. Isolation, enumeration, storage and characterization of *Vibrio* sp. isolates for future screening

When bacterial infections are suspected on the bases of clinical signs (Lightner, 1996), putative aetiological agents are isolated from diseased shrimps. Samples are taken aseptically from different tissues of juveniles to adult animals or from homogenized larvae and inoculated onto appropriate agar media using sterile seawater, NaCl (1–3% w/v) or artificial seawater as diluent. Trypticase soy agar, marine agar 2216 E (Difco) or nutritive agar medium (0.4% (w/v) peptone, 0.1% (w/v) yeast extract, pH 7.4) containing NaCl (1–3% w/v) are used to isolate bacteria or to determine the total heterotrophic aerobic bacterial counts in various shrimp samples as indicated above. Plates are then commonly incubated between 25°C and 30°C from 1 to 2 days and more precisely at the same temperature that the water where shrimps are reared and sampled.

For isolation, colonies, which are found to be the more abundant on the basis of their morphological characteristics, are streaked onto nutrient agar medium. *Vibriosp.* are generally enumerated using thiosulfate citrate bile salt sucrose (TCBS) agar and plates incubated 1 to 2 days as indicated above. Surprisingly, some *Vibrio*, including *V. penaeicida* strains isolated from New Caledonia, do not grow well on this media since bacteria enumeration is higher on marine agar using the same inoculum (Haffner and Goarant, personal communication). These results indicate that for some *Vibriosp.*, the number of *Vibrio* bacteria may be under estimated by the count plate method using TCBS agar-selective media.

Stock cultures of the bacterial strains may be stored at -80°C or under liquid nitrogen in marine broth to which 10–20% glycerol has been added to preserve pathogenicity. Our experience on shrimp challenges with New Caledonian *V. penaeicida* strains shows that the virulence of these strains diminish after 1 week of culture at $25-27^{\circ}\text{C}$ in nutritive agar medium (Saulnier, unpublished results).

Vibrio spp. are phenotypically characterized by standard morphological, physiological and biochemical tests (Baumann et al., 1984) and by using commercially available identification systems like the Biolog-GN system (BIOLOG, Hayward, CA, USA) or the Api 20E and API 20NFT kits (Bio Merieux, France). In order to obtain reproducible results when using commercial tests, bacterial isolates must be grown exclusively on tryptic soy agar. The metabolic fingerprints obtained can easily be compared to the existing database for bacterial identification. Nevertheless, complementary genotypical methods of characterization are sometimes essential such as the arbitrary fragment length polymorphism technique (Janssen et al., 1996; Vandenberghe et al., 1998), DNA–DNA hybridization assays (Costa et al., 1998), ribotyping (Grimont and Grimont, 1986; Aznar et al., 1993), arbitrarily primed polymerase chain reaction (Goarant et al., 1999), and random amplification of polymorphic DNA (Aznar et al., 1993).

3.2. Preparation of the inoculum and choice of the route of infection

Bacterial isolates are grown at constant temperature in trypticase soy broth (TSB), marine broth 2216 E (Difco) or nutritive medium containing 1–3% (w/v) NaCl for 12–24 h with constant shaking. The test inoculum is made by dilution of bacteria in sterile seawater, sterile normal saline solution (1–3% w/v NaCl) or sterile artificial seawater to produce the different doses of inoculum. Cell densities can be photometrically obtained at 590 nm or calculated using a Neubauer type haemocytometer but both are not very sensitive. Spreading the inoculum on nutrient agar plates is a more reliable technique because it allows determining the precise number of viable and culturable bacteria initially present in the inoculum. Our experience in challenge on the New Caledonian *V. penaeicida* strains shows that cultures reach a stationary phase after 10 h of incubation at 27°C under agitation with an air-to-medium ratio equal to three and that their virulence is not affected by the growth state of the bacteria either in stationary or exponential phase (Saulnier, unpublished results). Furthermore, the inoculum used in infection challenge must be prepared just before infection trials. *V. penaeicida* strains

appear to be very sensitive, in experimental conditions, to the lack of organic matter in the medium, and the number of cultivatable bacteria drops after 2 days of incubation at 25–30°C in artificial seawater. The decreasing virulence of such bacterial inocula could explain the fact that, in some studies, a relatively high number of bacteria are needed to produce the disease.

In shrimp, the different natural routes of infection by virulent bacterial isolates are theoretically oral, *trans*-cuticular or caused by wounds, by an imbalance in the natural bacterial flora, or by vertical transmission of the pathogen, even in this last case, and referring to the existing literature, there is no evidence that this way of infection may occur. In order to assess the virulence of newly isolated bacteria and to compare the virulence of different isolates, the use of such natural routes of infection should improve the reliability of any dose–response experimental infection. Unfortunately, these natural routes of infection have not been demonstrated for the majority of bacterial diseases. It is also probable that multiple routes of infection may occur simultaneously for the same pathogen. Nevertheless, the results of many epizootiological and ecological studies on the distribution of virulent strains suggest that a main source of infection is by waterborne bacteria (Lavilla-Pitogo et al., 1990, 1998; Lightner, 1992; de la Peña et al., 1992; Goarant et al., 1999). Thus, the immersion route of infection appears as a suitable method to experimentally test bacterial virulence. Furthermore, it allows a comparison of the LD50 of the used bacteria to natural concentrations in marine environment. By contrast, there are few reports about other possible natural routes of infection. de la Peña et al. (1995) succeeded in infecting *P. japonicus* prawns with low numbers of a *V. penaeicidai* isolate (10^3 – 10^4 CFU/animal) orally administered, whereas Lightner and Lewis (1975) found that the addition of virulent *V. alginolyticus* isolates to the shrimp feed was unsuccessful as a means of infection. Infecting *P. monodon* larvae by immersion with a *V. harveyi* strain, Lavilla-Pitogo et al. (1990) observed that colonization by the bacteria occurred specifically on the feeding apparatus and oral cavity of the larvae. Many virulence studies on animals of sufficient size to allow experimental injections have been reported using the IM way of infection between the fifth and sixth abdominal segments (see Table 1). These experiments could reproduce the natural bacterial disease that would occur after cuticular injuries, ecdysis or an imbalance in the natural bacterial flora.

3.3. Choice of animals, environmental parameters and the inoculum dose

The higher the number of challenged shrimps are, the more LD50 results of any pathogenicity tests are reliable. Replication of tanks for juveniles and adults (around 15 shrimp/m²) or bottles for larvae (20–50/l) encompassing experimentally challenged shrimp with various inoculum doses of the bacterial isolates, which are suspected to be true pathogens, control animals (batch of uninfected shrimp, batches of infected shrimp with other known pathogenic or nonpathogenic bacteria) need to be conducted in order to reduce the impact of some specific environmental factors. Furthermore, shrimp must be of the same age and species that those from which bacterial isolates have been isolated, healthy, acclimatized before experiments and kept in good conditions and at

densities comparable to the rearing conditions during all the period of survey. This last point includes the use of aerated seawater, high water quality obtained, for example, by filtration, ultraviolet or ozone treatments, poor nutrient content of the seawater to prevent the growth of bacteria or adsorption of *Vibrio* to organic matter, constant temperature and periodical water change. In the case of immersion challenge, a short time of contact (between 1 and 2 h) should be preferred in order to prevent possible bacterial reinfection during the experimental survey. At the end of this challenge, shrimp can be rinsed in high quality seawater to remove the waterborne pathogen and then transferred to clean seawater. A statistical analysis such as a χ^2 test would emphasize the effect of the inoculum dose and demonstrate for a same treatment the absence of any tank effect.

The inoculum dose depends on the route of infection selected (bath immersion vs. IM injection) and on the virulence of *Vibrio* isolates. Results of some pathogenicity tests using only *Vibrio* species isolated from diseased shrimp are given in Table 1. In all these experiments, animals have been challenged with serial dilutions of *Vibrio* suspensions in order to determine the lethal dose 50.

4. Conclusions

From this review, it should be evident that some *V. harveyi* and *V. penaeicida* strains isolated from moribund shrimp may be true pathogens and be the primary cause of disease. Results of immersion challenges with these isolates indicate that the *Vibriodensities* used for the calculation of the lethal dose 50 may be naturally encountered in the shrimp rearing water. This hypothesis is reinforced by the finding of Sung et al. (1999), who observed in seawater from *P. monodon* cultured pond a decrease in the diversity of the *Vibrio* community associated with a dominance of few potentially virulent *Vibrio* species prior to outbreaks of vibriosis. Similar results were obtained by Lavilla-Pitogo et al. (1998) monitoring the bacterial population in the rearing water of several ponds cultured *P. monodon*. A dominance of luminescent *Vibrio* in rearing water of infected ponds was observed prior to outbreaks due to luminescent *Vibrio*. Contrary to the immersion challenge, virulence studies using the IM way of infection are more difficult to analyze in terms of epidemiological significance.

There is a need for reproducible and standardized experimental models in order to evaluate the virulence of *Vibrio* isolates associated with mortalities, to test prophylactic and curative treatments and study the host-factors influencing the expression of bacterial virulence. As pointed out in this review, standardization of each step of pathogenicity tests is crucial because many environmental parameters, bacteria and shrimp factors may influence the results of a pathogenicity experiment.

References

- Alapide-Tendencia, E.V., Dureza, L.A., 1997. Isolation of *Vibrio spp.* from *Penaeus monodon* (Fabricius) with red disease syndrome. *Aquaculture* 154, 107–114.
- Alvarez, J.D., Austin, B., Alvarez, A.M., Reyes, H., 1998. *Vibrio harveyi*: a pathogen of penaeid shrimps and fish in Venezuela. *J. Fish Dis.* 21, 313–316.

- Anderson, I.G., Shamsudin, M.N., Shariff, M., Nash, G., 1988. Bacterial septicemia in juvenile tiger shrimp, *Penaeus monodon*, cultured in Malaysian brackishwater ponds. *Asian Fish. Sci.* 2 (1), 93–108.
- Aznar, R., Ludwig, W., Schleifer, K.H., 1993. Ribotyping and randomly amplified polymorphic DNA analysis of *Vibrio vulnificus* biotypes. *Syst. Appl. Microbiol.* 16, 303–309.
- Baticados, M.C.L., Lavilla-Pitogo, C.R., Cruz-Lacierda, E.R., de la Peña, L.D., Suñaz, N.A., 1990. Studies on the chemical control of luminous bacteria *Vibrio harveyi* and *V. splendidus* isolated from diseased *Penaeus monodon* larvae and rearing water. *Dis. Aquat. Org.* 9, 133–139.
- Baumann, P., Furniss, A.L., Lee, J.V., 1984. Genus *Vibrio* Pacini 1854, 411^{AL}. In: Krieg, N.R., Holt, J.G. (Eds.), *Bergey's Manual of Systematic Bacteriology* vol. 1 Williams and Wilkins, Baltimore, pp. 518–538.
- Colorni, A., Avtalion, R., Knibb, W., Berger, E., Colorni, B., Timan, B., 1998. Histopathology of sea bass (*Dicentrarchus labrax*) experimentally infected with *Mycobacterium marinum* and treated with streptomycin and garlic. *Aquaculture* 160 (1–2), 1–17.
- Costa, R., Mermoud, I., Koblavci, S., Morlet, B., Haffner, P., Berthe, F., Legroumellec, M., Grimont, P., 1998. Isolation and characterization of bacteria associated with a *Penaeus stylirostris* disease (Syndrome 93) in New Caledonia. *Aquaculture* 164, 297–309.
- de la Peña, L.D., Tamaki, T., Momoyama, K., Nakai, T., Muroga, K., 1992. Detection of the causative bacterium of vibriosis in Kuruma prawn, *Penaeus japonicus*. *Gyobyo Kenkyu* 27 (4), 223–228.
- de la Peña, L.D., Tamaki, T., Momoyama, K., Nakai, T., Muroga, K., 1993. Characteristics of the causative bacterium of vibriosis in the Kuruma prawn, *Penaeus japonicus*. *Aquaculture* 115, 1–12.
- de la Peña, L.D., Nakai, T., Muroga, K., 1995. Dynamics of *Vibrio* sp. in organs of orally infected Kuruma prawn, *Penaeus japonicus*. *Fish Pathol.* 30 (1), 39–45.
- Goarant, C., Régnier, F., Brizard, R., Marteau, A.L., 1998. Acquisition of susceptibility to *Vibrio Penaeicida* in *Penaeus stylirostris* postlarvae and juveniles. *Aquaculture* 169, 291–296.
- Goarant, C., Mérien, F., Berthe, F., Mermoud, I., Pérolat, P., 1999. Arbitrarily primed PCR to type *Vibrio* spp. pathogenic for shrimp. *Appl. Environ. Microbiol.* 65 (3), 1145–1151.
- Gram, L., Melchiorson, J., Spanggaard, B., Huber, I., Nielsen, T.F., 1999. Inhibition of *Vibrio anguillarum* by *Pseudomonas fluorescens* AH2, a possible probiotic treatment of fish. *Appl. Environ. Microbiol.* 65 (3), 969–973.
- Grimont, F., Grimont, P.A.D., 1986. Ribosomal ribonucleic acid gene restriction patterns as potential taxonomic tools. *Ann. Inst. Pasteur/Microbiol.* 137, 165–175.
- Hameed, A.S.S., 1995. Susceptibility of three *Penaeus* species to a *Vibrio campbellii*-like bacterium. *J. World Aquacult. Soc.* 26 (3), 315–319.
- Huang, M.T.F., Eble, A.F., Hammen, C.S., 1981. Immune response of the prawn, *Macrobrachium rosenbergii*, to bacterial infection. *J. Invertebr. Pathol.* 37, 213–219.
- Itami, T., Takahashi, Y., Nakamura, Y., 1989. Efficacy of vaccination against vibriosis in cultured Kuruma prawns *Penaeus japonicus*. *J. Aquat. Anim. Health* 1, 238–242.
- Janssen, P., Coopman, R., Huys, G., Swings, J., Blecker, M., Vos, P., Zabeau, P., Kersters, K., 1996. Evaluation of the DNA fingerprinting method AFLP as a new tool in bacterial taxonomy. *Microbiology* 142, 1881–1893.
- Jiravanichpaisal, P., Miyazaki, T., Limsuwan, C., 1994. Histopathology, biochemistry, and pathogenicity of *Vibrio harveyi* infecting black tiger prawn *Penaeus monodon*. *J. Aquat. Anim. Health* 6 (1), 27–35.
- Karunasagar, I., Pai, R., Malathi, G.R., Karunasagar, I., 1994. Mass mortality of *Penaeus monodon* larvae due to antibiotic resistant *Vibrio harveyi* infection. *Aquaculture* 128, 203–209.
- Katae, H., Kouno, K., Takase, Y., Miyazaki, H., Hashimoto, M., Shimizu, M., 1979. The evaluation of primidic acid as an antibiotic in fish: an in vitro and in vivo study. *J. Fish Dis.* 2 (4), 321–335.
- Lavilla-Pitogo, C.R., Baticados, M.C.L., Cruz-Lacierda, E.R., de la Peña, L.D., 1990. Occurrence of luminous bacterial disease of *Penaeus monodon* larvae in the Philippines. *Aquaculture* 91, 1–13.
- Lavilla-Pitogo, C.R., Leano, E.M., Paner, M.G., 1998. Mortalities of pond-cultured juvenile shrimp, *Penaeus monodon*, associated with dominance of luminescent *Vibrios* in the rearing environment. *Aquaculture* 164, 337–349.
- Lee, K.K., Yu, S.R., Chen, F.R., Yang, T.I., Liu, P.C., 1996. Virulence of *Vibrio alginolyticus* isolated from diseased tiger prawn, *Penaeus monodon*. *Curr. Microbiol.* 32, 229–231.

- Le Groumellec, M., Haffner, P., Martin, B., Martin, C., 1995. Comparative study of bacterial infections responsible for mass mortality in penaeid shrimp hatcheries of the Pacific zone. In: Shariff, M., Arthur, J.R., Subasinghe (Eds.), Diseases in Asian Aquaculture II. Fish Health Section, Asian Fisheries Society, Manila, pp. 163–173.
- Le Moullac, G., Le Groumellec, M., Ansquer, D., Froissard, S., Levy, P., AQUACOP, 1997. Haematological and phenoloxidase activity changes in the shrimp *Penaeus stylirostris* in relation with moult cycle: protection against vibriosis. Fish Shellfish Immunol. 7, 227–234.
- Lightner, D.V., 1988. *Vibrio* disease of penaeid shrimp. In: Sindermann, C.J., Lightner, D.V. (Eds.), Disease Diagnosis and Control in North American Marine Aquaculture Developments in Aquaculture and Fisheries Science vol. 17 Elsevier, Amsterdam, pp. 42–47.
- Lightner, D.V., 1992. Shrimp pathology: major diseases of concern to the farming industry in the Americas. Mem. Congr. Ecuat. Acuicult., 177–195.
- Lightner, D.V., Lewis, D.H., 1975. A septic bacterial disease syndrome of penaeid shrimp. Mar. Fish. Rev. 37, 25–28.
- Lightner, D.V., 1996. A Handbook of Pathology and Diagnostic Procedures for Diseases of Penaeid Shrimp. World Aquaculture Society, Baton Rouge, LA, 236 pp.
- Liu, P.C., Lee, K.K., Yui, K.C., Kou, G.H., Chen, S.N., 1996a. Isolation of *Vibrio harveyi* from diseased kuruma prawns *Penaeus japonicus*. Curr. Microbiol. 33, 129–132.
- Liu, P.C., Lee, K.K., Chen, S.N., 1996b. Pathogenicity of different isolates of *Vibrio harveyi* in tiger prawn, *Penaeus monodon*. Lett. Appl. Microbiol. 22, 413–416.
- Mermoud, I., Costa, R., Ferré, O., Goarant, C., Haffner, P., 1998. 'Syndrome 93' in New Caledonia outdoor rearing ponds of *Penaeus stylirostris*: history and description of three major outbreaks. Aquaculture 164, 323–335.
- Mohney, L.L., Bell, T.A., Lightner, D.V., 1992. Shrimp antimicrobial testing: I. In vitro susceptibility of thirteen gram-negative bacteria to twelve antimicrobials. J. Aquat. Anim. Health 4, 257–261.
- Mohney, L.L., Lightner, D.V., Bell, T.A., 1994. An epizootic of vibriosis in Equadorian pond-reared *Penaeus vannamei*. J. World Aquacult. Soc. 25, 116–125.
- Moriarty, D.W., 1998. Control of luminous *Vibrio* species in penaeid aquaculture ponds. Aquaculture 164, 351–358.
- Nakai, T., Nishimura, Y., Muroga, K., 1997. Detection of *Vibrio penaeicida* from apparently healthy Kuruma prawns by RT-PCR. Bull. Eur. Ass. Fish Pathol. 17 (3/4), 131–133.
- Pizzutto, M., Hirst, R.G., 1995. Classification of isolates of *Vibrio harveyi* virulent to *Penaeus monodon* larvae by protein profile analysis and M13 DNA fingerprinting. Dis. Aquat. Org. 21, 61–68.
- Prayitno, S.B., Latchford, J.W., 1995. Experimental infections of crustaceans with luminous bacteria related to *Photobacterium* and *Vibrio*. Effect of salinity and pH on infectivity. Aquaculture 132, 105–112.
- Rengpipat, S., Rukpratanporn, S., Piyatiratitvorakul, S., Menasveta, P., 1998. Probiotics in aquaculture: a case study of probiotics for larvae of the black tiger shrimp (*Penaeus monodon*). In: Flegel, T.W. (Ed.), Advances in Shrimp Biotechnology. Proceedings to the Special Session on Shrimp Biotechnology. 5th Asian Fisheries Forum, Chiangmai, Thailand. pp. 177–181.
- Robertson, P.A.W., Calderon, J., Carrera, L., Stark, J.R., Zherdmant, M., Austin, B., 1998. Experimental *Vibrio harveyi* infections in *Penaeus vannamei* larvae. Dis. Aquat. Org. 32, 151–155.
- Saulnier, D., Avarre, J.C., Le Moullac, G., Ansquer, D., Levy, P., Vonau, V., 2000. Evidence that *Vibrio penaeicida* is a putative etiological agent of syndrome 93 in New Caledonia and development of a rapid and sensitive PCR assay for its detection in shrimp and sea water. Dis. Aquat. Org. 40, 109–115.
- Song, Y.L., Lee, S.P., 1993. Characterization and ecological implication of luminous *Vibrio harveyi* isolated from tiger shrimp (*Penaeus monodon*). Bull. Inst. Zool., Acad. Sin. 32, 217–220.
- Song, Y.L., Cheng, W., Wang, C.H., 1993. Isolation and characterization of *Vibrio damsela* infectious for cultured shrimp in Taiwan. J. Invertebr. Pathol. 61, 24–31.
- Sung, H.H., Li, H.C., Tsai, F.M., Ting, Y.Y., Chao, W.L., 1999. Changes in the composition of *Vibrio* communities in pond water during tiger shrimp (*Penaeus monodon*) cultivation and in the hepatopancreas of healthy and diseased shrimp. J. Exp. Mar. Biol. Ecol. 236, 261–271.
- Sunaryanto, A., Mariam, A., 1986. Occurrence of pathogenic bacteria causing luminescence in penaeid larvae in Indonesia hatcheries. Bull. Br. Aqua. Dev. Center 8, 64–70.

- Takahashi, Y., Shimoyama, Y., Momoyama, K., 1985a. Pathogenicity and characteristics of *Vibriosp.* isolated from cultured kuruma prawn, *Penaeus japonicus* Bate. Bull. Jpn. Soc. Sci. Fish. 51, 721–730.
- Takahashi, Y., Itami, T., Nakagawa, A., Nishimura, H., Abe, T., 1985b. Therapeutic effects of oxytetracycline trial tablets against vibriosis in cultured Kuruma prawns *Penaeus japonicus* Bate. Bull. Jpn. Soc. Sci. Fish. 51, 1639–1643.
- Vandenberghe, J., Li, Y., Verdonck, L., Li, J., Sorgeloos, P., Xu, H.S., Swings, J., 1998. *Vibrio* associated with *Penaeus chinensis* (Crustacea: Decapoda) larvae in Chinese shrimp hatcheries. Aquaculture 169, 121–132.