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Host-induced increase in larval sea bass mortality in a gnotobiotic challenge test with *Vibrio anguillarum*

Xuan Li[1], Tom Defoirdt[1], Qian Yang[1], Stanislas Laureau[2], Peter Bossier[1,*], Kristof Dierckens[1]

[1]**Laboratory of Aquaculture & *Artemia* Reference Center, Ghent University, 9000 Gent, Belgium**
[2]**Ecloserie Marine de Gravelines, voie des Enrochements, 59820 Gravelines, France**

ABSTRACT: *Vibrio anguillarum* is the major cause of haemorrhagic septicaemia, vibriosis, which is a severe disease affecting marine fish. In this work, it was found that the mortality of gnotobiotic sea bass larvae challenged with *V. anguillarum* was dependent on the number of dead fish in the vials at the moment of challenge. Based on this finding, the effect of dead hosts (homogenised sea bass larvae or brine shrimp) on the virulence of *V. anguillarum* towards sea bass larvae was further investigated. Addition of homogenised hosts led to significantly increased larval mortality of challenged larvae, and this was observed for 3 different *V. anguillarum* strains, i.e. 43, NB 10 and HI 610. In contrast, the addition of similar levels of tryptone had no effect on mortality. In line with this, the motility of all 3 *V. anguillarum* strains was significantly increased by the addition of homogenised hosts but not by tryptone. These results suggest that dead hosts increase infectivity of *V. anguillarum,* not merely by offering nutrients to the bacteria, but also by increasing virulence-associated activities such as motility.

KEY WORDS: Vibriosis · Host–microbe interaction · Host–pathogen interaction

INTRODUCTION

Vibrio anguillarum is the causative agent of vibriosis, a fatal haemorrhagic septicaemia affecting a variety of fish species and other aquatic animals, and causing large economic losses in the aquaculture industry worldwide (Austin & Austin 2012). This is especially significant for European sea bass *Dicentrarchus labrax*, a species that is very sensitive to stressors and pathogens, and infections are often the major limiting factor for successful rearing (Torrecillas et al. 2007). A few virulence-related factors and genes have been identified in *V. anguillarum* (Frans et al. 2011), including genes affecting chemotaxis and motility (McGee et al. 1996, O'Toole et al. 1996), an iron uptake system (Crosa 1980, Stork et al. 2007), haemolytic activity (Rock & Nelson 2006), extracellular metalloprotease (Norqvist et al. 1990, Yang et al. 2007), lipopolysaccharides (Milton et al. 1995, Welch & Crosa 2005) and exopolysaccharides (Croxatto et al. 2007). However, the mechanisms of pathogenicity behind the disease caused by the bacterium are not yet completely understood.

As virulence factors produced by vibrios are often costly metabolic products, their production is generally tightly regulated (Ruwandeepika et al. 2012). Regulatory mechanisms that have been reported to control virulence gene expression in vibrios include quorum sensing and the ToxR regulon. However, neither of these has been found to affect the virulence of *Vibrio anguillarum* (Wang et al. 2002, Milton 2006). In addition to these regulatory mechanisms, host cues such as mucus (Hsiao et al. 2006), bile (Gotoh et al. 2010), bicarbonate (Abuaita & Withey 2009), catecholamine hormones and lipid hormones (Hughes & Sperandio 2008) are also known to affect virulence gene expression in pathogenic bacteria. However, as far as we know, the role of host cues in the pathogenicity of *V. anguillarum* has not yet been investigated.

*Corresponding author: peter.bossier@ugent.be

MATERIALS AND METHODS

Bacterial strains and culture conditions

Vibrio anguillarum strains 43 (B. Austin, Heriot-Watt University, Scotland), NB 10 (Milton et al. 1997) and HI 610 (O. Bergh, Institute of Marine Research, Norway) were used in this study. All strains were made rifampicin resistant by natural selection, allowing enumeration after introduction into the culture water of the axenic sea bass larvae. The bacteria were grown in 10% marine broth (Difco Laboratories) with the addition of Instant Ocean artificial sea salt (Aquarium Systems) to obtain a salinity of 36 g l^{-1} on a horizontal shaker (150 rpm) at 16°C for 48 h. The density of the bacterial suspensions was determined with a spectrophotometer (Genesys 20, Thermospectronic) at 550 nm according to the McFarland standard (BioMérieux).

Challenge tests with *Vibrio anguillarum* and gnotobiotic sea bass larvae

The disinfection of sea bass eggs, hatching and tests for axenity were performed according to Dierckens et al. (2009). On Day 0 after hatching (DAH 0), sea bass larvae were stocked in groups of 12 animals in 10 ml sterile screw cap vials with the addition of 10 mg l^{-1} rifampicin. Ten replicates were prepared for each treatment. *V. anguillarum* strain HI 610 was added to the sterile culture water on DAH 3 or DAH 7 at 10^5 CFU ml^{-1} (bath challenge). The survival was checked on DAH 3, 5, 7, 9 and 11 by counting the living sea bass larvae (transparent and swimming) under a dissecting microscope. Survival was monitored in the same vials for all the sampling points. The larvae were not fed during the experiment. Each experiment was repeated 3 times with a different batch of sea bass larvae. All tests in this study were approved by the ethical committee of Ghent University (EC2012/071).

Preparation of homogenised hosts

The axenic live sea bass larvae were killed using an overdose of benzocaine, rinsed 3 times with sterile artificial sea water (36‰) and homogenised using a sterile ground-glass tissue minihomogeniser. The homogenised fish suspension was kept frozen at −20°C until use. The homogenised brine shrimp were prepared in the same way.

Impact of homogenised hosts on the virulence of *Vibrio anguillarum* towards gnotobiotic sea bass larvae

Axenic larvae were stocked immediately before challenging (both on DAH 3). *V. anguillarum* strains 43, NB 10 and HI 610 were added to the culture water at 10^5 CFU ml^{-1} with and without the addition of 1 or 10 mg l^{-1} tryptone, or 3 mg l^{-1} homogenised sea bass larvae or brine shrimp *Artemia franciscana* (dry weight). Ten replicates were prepared for each treatment. The survival was checked on DAH 11. The larvae were not fed during the experiment.

Determination of the bacterial density associated with the fish larvae and the culture water

The bacterial density associated with the fish larvae and the culture water (in the experiment with the addition of 3 mg l^{-1} fish larvae or *Artemia*) was determined on DAH 7, 9 and 11. The sampling of live sea bass larvae and the culture water and the subsequent quantification of the bacterial density was performed according to Dierckens et al. (2009) with some modifications. Living sea bass larvae contaminated with *Vibrio anguillarum*, with and without the addition of 3 mg l^{-1} homogenised larvae (dry weight), were killed using an overdose of benzocaine, rinsed 3 times with sterile artificial sea water (36‰) and homogenised. Subsequently, the homogenised fish suspension and culture water were plated on 10% marine agar and incubated for 48 h at 28°C.

Virulence factor assays

Lipase, phospholipase, caseinase, gelatinase and haemolysin activities were assessed according to Natrah et al. (2011). Motility was assessed as described previously (Wang et al. 2011) on marine broth with 0.3% agar. Briefly, 2 µl of each *Vibrio anguillarum* overnight culture (optical density $[OD]_{550}$ = 0.5) was point inoculated in the middle of soft agar plates. After incubation for 24 h at 28°C, motility halos were measured. The impact of homogenised hosts was studied by adding the same density of homogenised fish or brine shrimp (3 mg l^{-1} dry matter) to the agar as used in the challenge tests. In an additional treatment, 6 mg l^{-1} tryptone (double amount of homogenised host dry weight) was used to test the effect of the nutrient level.

Statistics

The data on the sea bass tests and bacterial densities were analysed using independent samples *t*-tests. The data of virulence factor assays were analysed using 1-way ANOVA. All statistical analyses were done using the SPSS software version 19 at a significance level of 0.01.

RESULTS AND DISCUSSION

A challenge test with *Vibrio anguillarum* strain HI 610 and gnotobiotic sea bass larvae was performed according to Dierckens et al. (2009). In this set-up, variable mortality of sea bass larvae was observed in the vials between stocking (DAH 0) and the day of *V. anguillarum* bath challenge (DAH 7). Interestingly, these 'dead before challenge' larvae significantly affected the survival of sea bass larvae after challenge with strain HI 610. The mortality of the challenged sea bass larvae increased with increasing numbers of 'dead before challenge' larvae in the vials (Fig. 1A). In contrast, there was no relation between the mortality of non-challenged larvae between DAH 7 and DAH 11, and the number of dead larvae present in the vials on DAH 7 (Fig. 1B). These data are representative for 3 independent experiments. This observation was confirmed by another independent sea bass challenge test. In this test, the larvae were stocked on DAH 0 and bath-challenged with strain HI 610 on DAH 3. There was a positive correlation between the mortality of challenged sea bass larvae and the number of dead larvae before challenge (Fig. 1C), while there was no such relation in the non-challenged group (Fig. 1D). This increase in larvae mortality was significantly higher when the number of dead larvae before challenge was equal to or higher than 3.

Based on this finding, we set up a new challenge protocol to investigate the effect of dead hosts on the mortality of sea bass larvae due to *Vibrio anguillarum*. The sea bass larvae were stocked on DAH 3 and immediately challenged to avoid the uncontrolled presence of dead larvae before challenge. Three homogenised fish larvae per vial (3 mg l^{-1} dry matter) were added to simulate the presence of dead larvae. *V. anguillarum* has been reported to be pathogenic to brine shrimp *Artemia franciscana* (Defoirdt et al. 2005), and therefore homogenised brine shrimp (3 mg l^{-1} dry matter) was used as a second host potentially influencing the outcome of a *V. anguillarum* challenge. Tryptone, at different concentrations, was used as a control. Neither homogenised

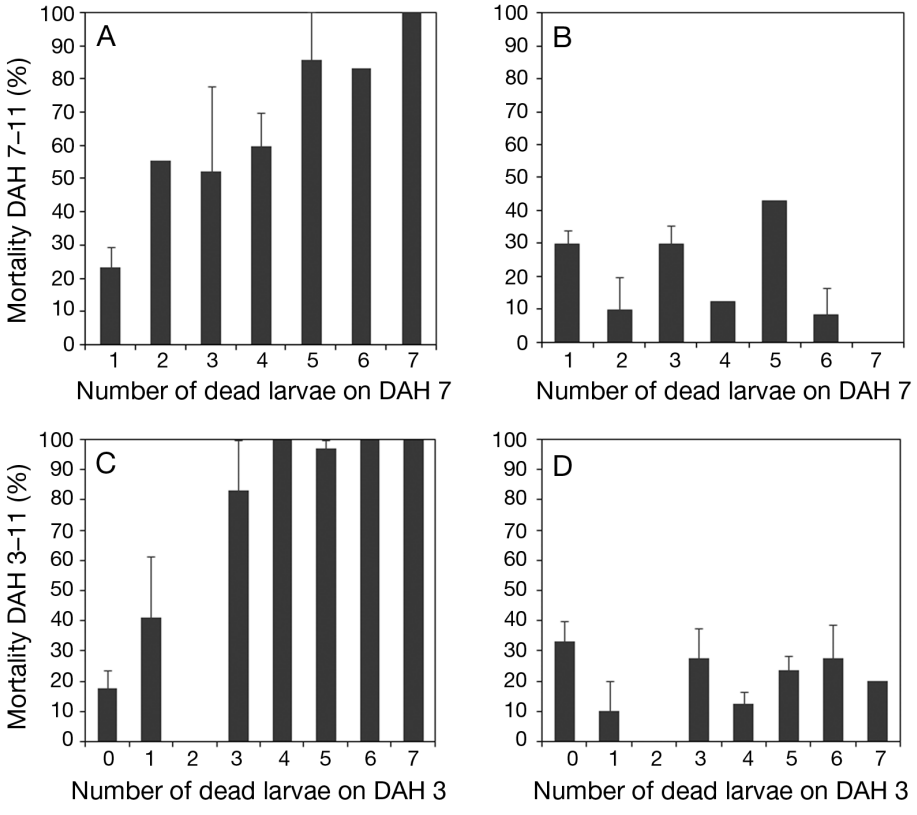

Fig. 1. *Dicentrarchus labrax* challenged with *Vibrio anguillarum*. (A,B) Mortality between Day 7 after hatching (DAH 7) and DAH 11 (A) of gnotobiotic sea bass larvae challenged with *V. anguillarum* HI 610 on DAH 7 and (B) of axenic larvae, as a function of the number of dead larvae present in the vial on DAH 7, and (C,D) mortality between DAH 3 and DAH 11 (C) of gnotobiotic larvae challenged with *V. anguillarum* HI 610 on DAH 3 and (D) of axenic larvae, as a function of the number of dead larvae present in the vial on DAH 3. Error bars represent the standard error. These data are representative for 3 independent experiments

hosts nor different concentrations of tryptone had any effect on the survival of unchallenged sea bass larvae. Strains 43, NB 10 and HI 610 did not cause significant mortality when added to the culture water at 10^5 CFU ml^{-1} in the absence of any other addition. However, in the presence of homogenised fish or brine shrimp, all 3 strains caused significant mortality (Table 1). Strain 43 was defined as avirulent towards sea bass larvae in a previous study (Dierckens et al. 2009). However, the addition of homogenised fish together with strain 43 resulted in significant mortality of the sea bass larvae.

Together, these experiments (Fig. 1, Table 1) demonstrate that sea bass larval mortality after bath challenge with *Vibrio anguillarum* is affected by the presence of low levels of host substances, as none of the strains was virulent in the absence of homogenised or dead larvae, whereas they were virulent in their presence. Hence, the addition of homogenised fish at the moment of challenge constitutes an experimental improvement, as it makes the outcome of the challenge independent from the presence of dead larvae.

The addition of 3 mg l^{-1} organic matter in the form of homogenised fish larvae or brine shrimp constitutes a source of nutrients for the added pathogens. This was clearly confirmed by the increase in *Vibrio*-CFU in the sea bass culture water (Table 2). At the majority of sampling points (6 out of 7 cases), independently of the strain used, the bacterial density was significantly higher in the treatments receiving homogenised fish or brine shrimp. The increase in bacterial density in the treatments receiving homogenised fish or brine shrimp was less strong for the fish larvae-associated bacteria (only 2 out of 7 cases).

With the addition of homogenised host (3 mg l^{-1} dry matter) in sterile artificial sea water, *Vibrio anguillarum* strains 43, NB 10 and HI 610 grew from an initial density of 10^5 CFU ml^{-1} to approximately 10^6 CFU ml^{-1}. A similar increase in *V. anguillarum* density was observed after addition of 10 mg l^{-1} tryptone. Thus, a higher number of bacteria can be ruled out as the sole reason for the higher mortality.

As host cues are also known to affect virulence gene expression in pathogenic bacteria, in a final series of experiments, we investigated whether homogenised hosts had an effect on virulence factor activities of the vibrios. The addition of homogenised fish or brine shrimp significantly increased the motility of all 3 *Vibrio anguillarum* strains on soft agar plates (Table 3). The addition of 6 mg l^{-1} tryptone (i.e. double the amount of dry matter when compared to the homogenised host treatments), in contrast, did not affect the motility. This result suggests that the

Table 1. *Dicentrarchus labrax* challenged with *Vibrio anguillarum*. Relative percentage survival (RPS) of gnotobiotic sea bass larvae on Day 11 after hatching (DAH 11) challenged with *V. anguillarum* strains (mean ± SE of 10 replicates). RPS = 1 − (% mortality in the challenge with treatment group/% mortality in the challenge group) × 100. Fish and shrimp homogenates were added at 3 mg l^{-1} dry weight. $^{**}p < 0.01$ as compared with corresponding challenge control value

Treatment	RPS
Control	100 ± 0
1 mg l^{-1} tryptone	104 ± 5
10 mg l^{-1} tryptone	109 ± 11
Homogenised fish	89 ± 4
Homogenised brine shrimp	99 ± 3
NB 10	100 ± 0
NB 10 + 1 mg l^{-1} tryptone	103 ± 8
NB 10 + 10 mg l^{-1} tryptone	82 ± 9
NB 10 + homogenised fish	24 ± 5**
NB 10 + homogenised brine shrimp	35 ± 2**
HI 610	100 ± 0
HI 610 + 1 mg l^{-1} tryptone	80 ± 13
HI 610 + 10 mg l^{-1} tryptone	75 ± 13
HI 610 + homogenised fish	18 ± 12**
HI 610 + homogenised brine shrimp	0 ± 0**
43	100 ± 0
43 + homogenised fish	41 ± 13**

Table 2. *Vibrio anguillarum* infecting *Dicentrarchus labrax*. Density of the *V. anguillarum* strains 43, NB 10 and HI 610 associated with live sea bass larvae and in the larval culture water (mean ± SE of 3 fish cultures), with and without addition of homogenised fish or brine shrimp to the culture water on Day 3 after hatching (DAH 3). The larvae were challenged on DAH 3 by adding the strains to the culture water at 10^5 CFU ml^{-1}. The samples were from the experiment reported in Table 1. HF: homogenised fish (3 mg l^{-1} dry weight); NA: not available. *Significantly different from the corresponding treatment without homogenised fish ($p < 0.05$)

Time point (DAH)	Larvae log(CFU $larva^{-1}$) Control	+ HF	Culture water log(CFU ml^{-1}) Control	+ HF
***V. anguillarum* 43**				
7	NA	4.5 ± 0.1	NA	6.1 ± 0.1
9	4.4 ± 0.0	4.4 ± 0.5	5.2 ± 0.4	6.0 ± 0.2*
11	4.4 ± 0.4	3.5 ± 0.3	5.8 ± 0.1	6.3 ± 0.3*
***V. anguillarum* NB 10**				
7	4.1 ± 0.1	5.6 ± 0.2*	5.9 ± 0.4	7.2 ± 0.1*
9	4.5 ± 0.5	5.1 ± 0.1	7.3 ± 0.0	7.3 ± 0.1
11	4.8 ± 0.1	4.8 ± 0.5	6.7 ± 0.0	7.1 ± 0.2*
***V. anguillarum* HI 610**				
7	NA	5.4 ± 0.1	4.6 ± 0.0	6.3 ± 0.1*
9	3.6 ± 0.0	5.4 ± 0.1*	NA	6.7 ± 0.1
11	3.6 ± 0.3	4.1 ± 0.2	6.0 ± 0.2	7.1 ± 0.1*

Table 3. *Vibrio anguillarum*. Motility of strains 43, NB 10 and HI 610, with and without the addition of tryptone, homogenised fish or homogenised brine shrimp (mean ± SE of 3 replicates). Values in the same column with different superscript letters are significantly different ($p < 0.05$)

Treatment	Motility halo (mm)		
	43	NB 10	HI 610
Control	29.7 ± 1.2^{a}	34.0 ± 2.1^{A}	48.7 ± 1.9^{x}
Tryptone 6 mg l^{-1}	30.0 ± 2.9^{a}	39.3 ± 1.8^{A}	49.7 ± 0.9^{x}
Homogenised fish (3 mg l^{-1} dry weight)	56.3 ± 1.9^{b}	59.0 ± 0.6^{B}	68.0 ± 1.5^{y}
Homogenised brine shrimp (3 mg l^{-1} dry matter)	59.0 ± 0.6^{b}	63.3 ± 1.7^{B}	69.3 ± 1.2^{y}

increase in motility on plates with homogenised hosts was not due to increased growth of the bacteria caused by the extra nutrients added through the homogenised hosts. However, other virulence factors (including lipase, phospholipase, caseinase, gelatinase and haemolysin activities) were not affected by the addition of homogenised hosts (data not shown).

Studies have shown that for many pathogens, motility is essential in some phases of their life cycle and that virulence and motility are often intimately linked by complex regulatory networks (Josenhans & Suerbaum 2002). *Vibrio anguillarum* uses motility to colonise its aquatic hosts, which is essential for this bacterium to cause disease (Weber et al. 2010). Non-motile *V. anguillarum* mutants have been constructed and were found to be avirulent in a bath challenge, whereas infection via intraperitoneal injection showed no loss in virulence (O'Toole et al. 1996). The importance of motility for transmission and invasion has been corroborated using mutants defective in producing the major flagellin FlaA (Milton et al. 1996). Ormonde et al. (2000) showed that motility aids invasion by *V. anguillarum*, both *in vivo* and *in vitro*. Therefore, our results suggest that the larval mortality in treatments receiving homogenised fish or brine shrimp was not only due to an increased bacterial density but also to increased virulence-associated activity of the *V. anguillarum* strains. Similar results have been reported for *V. cholerae*, where cells shed from the human gastrointestinal tract showed greatly enhanced infectivity, which led the authors to hypothesise that epidemics may be propagated by the human host (Merrell et al. 2002). This increased infectivity was linked to high expression levels of genes required for nutrient acquisition and motility. Substances present in homogenised fish or brine shrimp that have been previously reported to enhance motility in pathogenic bacteria include mucus (Burghoff et al. 1993, Hsiao et al. 2006), catecholamine hormones, i.e. norepinephrine (Hegde et al. 2009), and lipid hormones, i.e. cholesterol (Chatterjee et al. 2007, Gilk et al. 2013). These factors might be responsible for the increased virulence of the strains in the treatments receiving homogenised hosts.

CONCLUSION

We have shown that under highly controlled conditions, the virulence of *Vibrio anguillarum* strains HI 610, NB 10 and 43, and thereby the outcome of a gnotobiotic *V. anguillarum* bath challenge, are dependent on the presence of host substances. In order to accommodate for this, an improved experimental set-up was developed in which the vibrios were added to the culture water at 10^5 CFU ml^{-1} on DAH 3 together with homogenised fish (added at 3 mg l^{-1}, equivalent to 3 larvae per 10 ml). *In vitro* observations have shown that specific, but as yet uncharacterised, host cues present in the homogenised hosts strongly increase motility of *V. anguillarum*, suggesting that the increased mortality is (at least partly) due to a host-induced increase in virulence. Our study indicates that, in practice, weak fish and live feed not only serve as a vector for pathogens, but can actually make pathogens more virulent.

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