Aquaculture 435 (2015) 28–32

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Short Communication

Characterization of the virulence of *Harveyi* clade vibrios isolated from a shrimp hatchery in vitro and in vivo, in a brine shrimp (*Artemia franciscana*) model system

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ARTICLE INFO

Article history:
Received 1 August 2014
Received in revised form 5 September 2014
Accepted 10 September 2014
Available online 16 September 2014

Keywords:
Artemia franciscana
Shrimp
Vibrio spp.
Virulence factors

ABSTRACT

Vibrios belonging to the *Harveyi* clade are important pathogens of a large number of marine animals in the aquaculture industry. In this study, six isolates (H1 to H6) were obtained from a shrimp hatchery in Rio Grande do Norte (Natal-Area, Brazil), which had been confronted with disease outbreaks in 2009. The aim was to characterize the virulence of these isolates, both in vitro (virulence factor production) and in vivo (virulence towards gnotobiotic brine shrimp, *Artemia franciscana*, larvae) and to compare these characteristics to those of the most virulent pathogen in the brine shrimp model described to date, *Vibrio campbellii* LMG 21363. Of all 6 isolates, H5 and H6 were found to be the most virulent ones and were therefore selected for further characterization. Isolate H5 exhibited a similar virulence as the control strain, while H6 exhibited a higher virulence, both in gnotobiotic and conventionally reared brine shrimp. Both H5 and H6 were motile and produced all of the lytic enzymes tested (hemolysin, caseinase, gelatinase, lipase and phospholipase). Although H6 was the most virulent isolate in vivo, this was not reflected in the highest production of all virulence factors tested. Finally, isolates H5 and H6 were identified to belong to the *Harveyi* clade of vibrios.

1. Introduction

Vibrios are Gram-negative bacteria that are ubiquitous in the marine environment and can be found free-living in the water column, as part of a biofilm, or in association with a host (Thompson et al., 2004). The *Harveyi* clade of vibrios is a subgroup containing major aquaculture pathogens (Ruwandeepika et al., 2012). Vibriosis causes severe economical losses in shrimp, finfish and mollusk cultivation worldwide (Austin and Zhang, 2006; FAO, 2012; Defoirdt et al., 2007).

The pathogenicity mechanisms of vibrios belonging to the *Harveyi* clade remain largely unraveled; however some virulence indicators have been described. The infectious cycle of pathogenic bacteria includes entry of the pathogen, establishment and multiplication, thereby causing damage to host tissues and cells, and exit (Donnenberg, 2000). These different steps involve the expression of virulence factors — gene products that allow the pathogens to infect and damage the host (Defoirdt, 2013). The ability to colonize and adhere to host surfaces is an essential step in successful infection of a host, and flagellar motility is thought to enhance the initial interaction of a bacterium with a surface by enabling the cell to overcome negative electrostatic forces (McCarter, 2004). Hence, motility can give an indication of the potential of an isolate to colonize a host. A second important phenotype in colonization of the host is biofilm formation (Costerton et al., 1981). The biofilm formation in vibrios depends on several factors including flagella, pili and exopolysaccharide biosynthesis (Yildiz and Visick, 2009). Exopolysaccharides form a loose slime outside the cell that forms an intercellular matrix in biofilms, which enhances the growth and survival of microorganisms by providing access to nutrients and protection from detergents or antimicrobials, predators and drying (Donlan and Costerton, 2002). A third group of virulence factors includes lytic enzymes, which are produced by many pathogenic bacteria and often play a central role in pathogenesis (Finlay and Falkow, 1997). These enzymes cause damage to host tissues, thereby allowing the pathogen to obtain nutrients and to spread through tissues. Lytic enzymes produced by pathogenic vibrios include hemolysins, proteases (including caseinase and gelatinase), and (phospho) lipases (Defoirdt, 2013).

In 2009, 6 *Vibrio* strains (H1-6) were isolated from a shrimp hatchery in Rio Grande do Norte (Natal-Area, Brazil), where high mortalities were faced at that moment. In this study, the aim was to characterize the virulence of these isolates, by determining the production of some important virulence factors in vitro, and by determining their virulence towards gnotobiotic and conventionally reared brine shrimp (*Artemia*

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http://dx.doi.org/10.1016/j.aquaculture.2014.09.015
0044-8486/

franciscana), a well-established model organism with respect to infections caused by *Vibrio* species in crustaceans (Soto-Rodriguez et al., 2003; Austin et al., 2005; Defoirdt et al., 2006).

2. Materials & methods

2.1. Bacterial strains & growth conditions

The isolates H1-6 were obtained from a hatchery in Rio Grande do Norte in 2009 and were originally isolated from pacific white shrimp *Penaeus vannamei* (Natal-Area, Brazil). The *Vibrio campbellii* strain LMG 21363 and the six isolates were stored in 30% glycerol at −80 °C. All bacterial strains were grown in Marine Broth (Difco) at 28 °C with constant agitation. Bacterial densities were determined spectrophotometrically at 550 nm. The bacterial densities were calculated according to the McFarland standard (BioMerieux, Marcy L'Etoile, France), assuming that an $OD_{550} = 1.000$ corresponds to 1.2×10^9 cells/ml.

2.2. Identification of the bacterial strains H5 and H6

A partial 16S rRNA gene sequence was amplified for strains H5 (1135 bp) and H6 (988 bp) as described previously (Castro et al., 2013). The conserved primers pA (5′ AGAGTTTGATCCTGGCTCAG 3′) and pH (5′ AAGGAGGTGATCCAGCCGCA 3′) were used to obtain the amplification products, while the primers *Gamma, Gamma and BKL1 (Coenye et al., 1999; Cleenwerck et al., 2007) were used to obtain the sequences. The partial sequences of both strains were compared with nearly complete 16S rRNA gene sequences of the type strains of the established *Vibrio* species retrieved from the EMBL database using the BioNumerics 5.1 software (Applied Maths, Belgium). A phylogenetic tree was constructed with the BioNumerics 5.1 software (Applied Maths) using the Neighbor-Joining method (Saitou and Nei, 1987).

2.3. Gnotobiotic culture of brine shrimp & challenge tests

All experiments were performed with *Artemia franciscana* cysts, originating from Great Salt Lake, Utah, USA (INVE Aquaculture NV, Belgium). Bacteria-free cysts and larvae were obtained via decapsulation according to the procedure described by Sorgeloos et al (1986). During decapsulation aeration was provided through a 0.22-μm filter. All procedures were performed under a laminar flow hood to maintain axenic conditions. All equipment was previously sterilized and autoclaved at 120 °C for 20 min. Decapsulated cysts were washed several times over a 100 μm sieve with sterile Instant Ocean (35 g l^{-1}) and carefully capped. The tubes were placed on a rotor at four cycles per minute and constantly exposed to an incandescent light at 28 °C for 18–22 h. Groups of 20 larvae (instar II stage nauplii) were collected and transferred to sterile glass tubes with filtered autoclaved seawater (35 g l^{-1}).

Isolates of the bacterial strains were aseptically inoculated in 30 ml Difco™ Marine Broth 2216 and incubated overnight at 25°–28 °C with constant agitation. 150 μl was subsequently transferred and grown to stationery phase in 30 ml marine broth 6 h before challenge. The bacterial densities were determined spectrophotometrically at an optical density of 550 nm. The challenge test was performed according to Marques et al. (2006), no feed was added. The bacterial suspension was added at a density of 10^7 cells ml^{-1}. Each treatment consisted out of 4 replicates. The survival of the brine shrimp larvae was determined after 48 h according to Amend (1981). The relative percentage of survival (RPS) was calculated as follows:

$$\text{RPS}\ (\%) = (\%\ \text{of surviving challenged larvae}) / (\%\ \text{of surviving unchallenged larvae}) \times 100.$$

Axenity of non-challenged brine shrimp cultures was verified by transferring 100 μl of culture medium to petri dishes containing Marine Agar 2216 (n = 3). Plates were stored in an incubator for five days at 28 °C. Only experiments of which the non-challenged cultures were confirmed to be axenic were considered for analysis.

2.4. Conventional culturing of brine shrimp & challenge tests

The same *Artemia franciscana* cysts as the gnotobiotic setup were used in the conventional experiments. Cysts were hatched in saltwater with strong aeration and constant illumination at 28 °C during 18–22 h. Groups of 20 larvae (instar II stage nauplii) were collected and transferred to glass tubes with saltwater for the duration of the challenge test. The conventional challenge test was performed in the same way as previously described for the gnotobiotic challenge. Each treatment consisted out of 4 replicates.

2.5. Virulence factor assays

Swimming motility and the production of the virulence factors caseinase, gelatinase, lipase, hemolysin and phospholipase were determined as described by Yang and Defoirdt (2014) and Natrah et al. (2011). The motility assay was done using soft agar plates (2% agar) with LB medium (10 g NaCl). The diameter of the swimming zone was measured after 24 h incubation at 28 °C. For the lipase and phospholipase assays, marine agar plates were supplemented with 1% Tween 80 (Sigma-Aldrich) or 1% egg yolk emulsion (Sigma-Aldrich), respectively. The development of opalescent zones around the colonies was observed and the diameter of the zones was measured after 2–4 days of incubation at 28 °C. The caseinase assay plates were prepared by mixing double strength MA with a 4% skim milk powder suspension (Oxoid, Basingstoke, Hampshire, UK), sterilized separately at 121 °C for 5 min. Clearing zones surrounding the bacterial colonies were measured after 2 days of incubation at 28 °C. Gelatinase assay plates were prepared by mixing 0.5% gelatin (Sigma-Aldrich) into MA. After incubation for 7 days, saturated ammonium sulfate (80%) in distilled water was poured over the plates and after 2 min, the diameters of the clearing zones around the colonies were measured. Hemolytic assay plates were prepared by supplementing MA with 5% defibrinated sheep blood (Oxoid) and clearing zones were measured after 2 days of incubation at 28 °C. All treatments were performed in triplicate.

2.6. Statistical analysis

Data analysis was done by independent samples t-tests or ANOVA with a Duncan post hoc analysis. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS), version 21.0 using a significance level of 5%.

3. Results & discussion

3.1. Virulence of the isolates towards brine shrimp (A. franciscana) larvae

In a first experiment, we investigated the virulence of the 6 isolates towards gnotobiotic brine shrimp larvae. *V. campbellii* LMG 21363, as far as we know the most virulent isolate in the brine shrimp model reported to date, was used as a control strain. All isolates were found to cause significant mortality (Fig. 1). Isolate H3 was significantly less virulent than the other strains. Strains H5 and H6 were significantly more virulent than the other isolates, with H6 tending to be more virulent than the control strain LMG 21363 (although the difference was not significant in this analysis). We decided to focus on isolates H5 and H6 in further experiments because they were the most virulent.

In a second experiment, we further investigated the virulence of isolates H5 and H6 towards conventionally reared brine shrimp larvae. *V. campbellii* LMG 21363 was again used as control strain. H6 showed the highest mortality and statistical analysis showed that mortality