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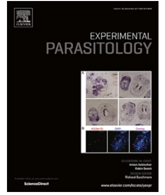

In vitro interactions of *Acanthamoeba castellanii* Neff and *Vibrio harveyi*

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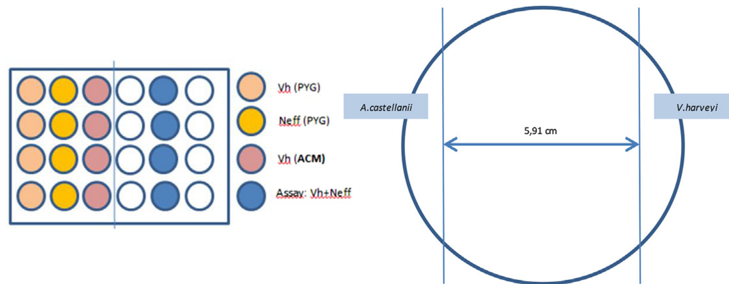
HIGHLIGHTS

- Vibrio harveyi culturability was not significantly improved after incubation with Acanthamoeba castellanii Neff.
- Acanthamoeba castellanii Neff seems to present a chemoattraction to Vibrio harveyi inoculum.
- Acanthamoeba could play a role as vehicle of Vibrio harveyi in the environment.

GRAPHICAL ABSTRACT



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ABSTRACT

Free-living amoebae (FLA) are opportunistic protozoa widely distributed in the environment. They are frequently found in water and soil samples, but they have also been reported to be associated with bacterial human pathogens such as *Legionella* spp. *Campylobacter* spp or *Vibrio cholerae* among others. Including within *Vibrio* spp. *V. harveyi* (Johnson and Shunk, 1936) is a bioluminescent marine bacteria which has been found swimming freely in tropical marine waters, being part of the stomach and intestine microflora of marine animals, and as both a primary and opportunistic pathogen of marine animals. Our aim was to study the interactions between *Vibrio harveyi* and *Acanthamoeba castellanii* Neff. Firstly, in order to analyze changes in it cultivability, *V. harveyi* was coincubated with *A. castellanii* Neff axenic culture and with *Acanthamoeba* Conditioned Medium (ACM) at different temperatures in aerobic conditions. Interestingly, at 4 °C and 18-20 °C bacteria were still cultivable in marine agar, at 28 °C, in aerobic conditions, but there weren't significant differences comparing with the controls. We also noted an enhanced migration of *Acanthamoeba* toward *V. harveyi* on non-nutrient agar plates compared to controls with no bacteria.

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1. Introduction

Some Free-Living Amoebae (FLA) are able to act as

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opportunistic pathogens but also as vehicles of other pathogens such as bacteria and viruses (Balczun and Scheid, 2017; Pagnier et al., 2015; Scheid, 2014; Thomas et al., 2010). It has been reported the significant association between *Legionella* spp. and *Mycobacterium* spp. with FLA in different drinking water systems (Corsaro and Venditti, 2010; Thomas et al. 2006). Bacteria which will be able to avoid amoeba digestion will gain a shelter where they could be able to proliferate and be protected from hostile external conditions (Strassmann and Shu, 2017; Guimaraes et al., 2016; Casadevall, 2008; Hilbi et al., 2007; Axelsson et al., 2005; Greub and Raoult, 2004). Currently, there are five described FLA groups which are able to produce pathologies in humans and other animals: *Acanthamoeba* spp. *Balamuthia mandrillaris*, *Naegleria fowleri*, *Sappinia pedata* and *Vermamoeba vermiformis* (Aitken et al., 1996; Brieland et al., 1997; Greub and Raoult, 2004; Visvesvara et al., 2007; Qvarnstrom et al., 2009). Regarding the prevalence in water bodies, FLA of *Acanthamoeba* and *Vermamoeba* genus are among the most common genera/species described in the literature (Fouque et al., 2015; Richards et al., 2013; Lorenzo-Morales et al., 2007, 2015).

The aquaculture is an aquatic organisms farming which started in China 3800 years BC, and it has been used as an alternative to conventional fishing (Pang and Pauly, 2001). It started in the Canary Islands at the end of the 80′s, specifically in the two capital islands, Gran Canaria and Tenerife (GEVIC-NACUL, 2002). The archipelago marine temperature benefits the marine organisms' growth. But one of the most important factor which affects aquaculture around the world is the incidence of microbial pathologies, mainly produced by bacteria. The ubiquitous luminescent gram-negative bacteria *Vibrio harveyi* is the most frequently isolated marine Vibrio species. It exists as a free-living bacteria as well as commensal pathogen in the enteric guts of marine animals (Austin and Zhang, 2006; Karunasagar et al., 1994). *V. harveyi* is able to infect the damaged tissues and lead to a serious, sometimes fatal infection. This pathogen is frequently found contaminating seafood and causing severe foodborne disorders in several Asian countries including China, Japan, India and Taiwan (Srinivasan et al., 2017). Undercooked contaminated-seafood can cause acute gastroenteritis (Drake et al., 2007) and produce massive deaths and dramatic economic loss for the culture industry (Harikrishnan et al., 2011).

V. harveyi possesses different (Johnson and Shunk, 1936) sets of virulence determinants which allow its attachment to the host tissues, evade the immune system and spread infection (Srinivasan et al., 2017). Some important virulence characteristics are the production of extracellular enzymes such as protease, chitinase, siderophore and, as the most important, biofilm formation. A biofilm is an assemblage of surface-associated microbial cells that is enclosed in an extracellular self-secreted exopolysaccharides (EPS) matrix. These biofilms offer a protective environment for bacteria, preventing the action of antimicrobials and even evading host defense mechanisms (Sethupathy et al., 2016). Biofilm formation contribute to the establishment of FLA cultures in the environment (Preston et al., 2001; Khan, 2006; Scheid et al., 2008) and they are formed in the "air-water" interface or water surrounded (Preston et al., 2001), increasing significantly the cell attachments (Davies et al., 1981). Besides, the biofilm formation may also increase their uptake by biofilm amoebae (Abdel-Nour et al., 2014), leading to a higher likelihood of selecting for virulent biofilm community members (Lau and Ashbolt, 2009).

The main objective of this work has been to elucidate if there is a relation between *V. harveyi* and the opportunistic protozoan *Acanthamoeba castellanii* Neff. We also evaluated *A. castellanii* movement in presence of *V. harveyi* inoculum.

2. Materials and methods

2.1. Acanthamoeba culture

The reference strain *Acanthamoeba castellanii* Neff (ATCC 30010, genotype T4) was used in this study. It was grown axenically in PYG medium (0.75% [wt/vol] proteose peptone, 0.75% [wt/vol] yeast extract, 1.5% [wt/vol] glucose) (Martín-Navarro et al., 2008) with no added antibiotics and supplemented with sea salts (15%). The amoebae were grown in tissue culture flasks at 30 °C without shaking.

2.2. Bacterial cultures

Vibrio harveyi had been grown on conventional marine agar plates in aerobic conditions at 28 °C for 24 h. 1 colony was suspended in fresh PYG medium supplemented with sea salts (15%), and was grown at 28 °C in aerobic conditions for 16 h. The resulting cell concentration was determined optically using a Bürker chamber (Axelsson-Olsson et al., 2005).

2.3. Antibiotic sensitivity assays

Different *V. harveyi* cultures were incubated in rotation (70 rpm) in PYG with 10 mg/ml of Gentamicine to evaluate its antibiotic susceptibility. 100 µl of each culture were seeded in marine agar plates at 4, 8 and 24 h which were incubated at 28 °C for 24 h 10^4 cells/ml of *A. castellanii* Neff were incubated with the same antibiotic at the same concentration. After 4, 8, and 24 h of incubation the amoebae growth was checked using light microscopy.

2.4. Culturability assays

The coculture was carried out in 24 well plates and it was performed by adding 1 ml of *V.harveyi* 10^7 colonies forming unit (cfu)/ml grown in PYG solution supplemented with sea salts (15%) to the culture plate wells where 1 ml of *A. castellanii* Neff 10^6 amoebas/ml had been incubated for 1 h, resulting in a cell ratio of amoebae to *V.harveyi* of approximately 1:10. 1 ml of *V.harveyi* 10^7 cfu/ml was incubated in presence of 1 ml of *Acanthamoeba* Conditioned Medium (ACM) (Axelsson-Olsson et al., 2005). As a negative control, 1 ml of *V. harveyi* 10^7 fcu/ml with 1 ml of PYG supplemented with sea salts (15%)was used. 1 ml of *A. castellanii* Neff 10^6 amoebas/ml with 1 ml of PYG medium supplemented with sea salts (15%) was incubated as an amoebae control. After inoculation, the cocultures were incubated in aerobic conditions at four different temperatures, 4, 18-20, 30, and 37 °C. Each 24 h, 100 µl of supernatant from cocultures, ACM-*V.harveyi* and negative control were seeded in a marine agar plate (aerobic environment, 28 °C, 24 h) to evaluate the presence of *V.harveyi*.

2.5. Evaluation Acanthamoeba castellanii Neff movement

To develop the evaluation of *A. castellanii* movement assay, we have used 90 × 14.2 mm no nutrient agar plates (Fig. 1). A 10^6 cells/ml of *A. castellanii* Neff solution was seeded on one of the petri plate side and the inoculum of 10^7 cfu/ml of *V. harveyi* was seeded in the opposite side. To evaluate the movement from the origin, the plates were marked with a graduated scale and 5,91 cm were taken as the total distance to be completed (Fig. 1). These plates were incubated at 28 °C and checked daily under the microscope in order to measure the distance and the trajectory of the amoeba. As a negative control the same assay was carried out with plates with no *V. harveyi* inoculum on the opposite side from amoeba culture.

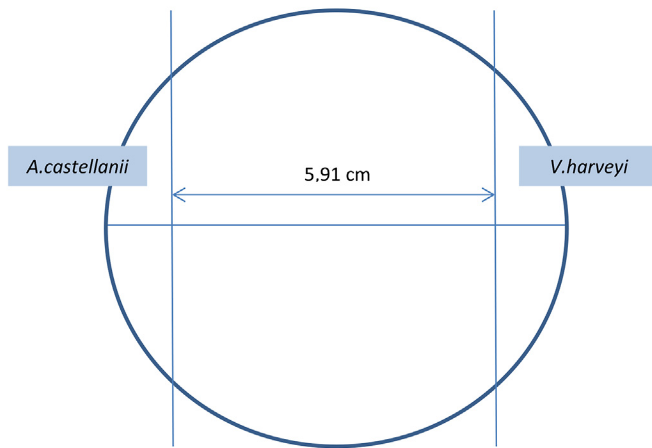

Fig. 1. Diagram of no nutrient agar plates used to evaluate the distance covered by amoeba.

3. Results

3.1. Culturability assays

At 4 °C, the growth of *V.harveyi* in *V.harveyi-Acanthamoeba* coculture has been maintained for more than 20 days. Interestingly, the negative control was cultivable for almost 25 days. In ACM-*V. harveyi* culture, colonies were detected just for 17 days. At 18-20 °C, ACM-*V. harveyi* culture and the negative control were only grown for 9 and 8 days respectively. On the contrary, *V. harveyi* from the coculture grew until more than 11 days. At 48 h and at temperature of 30 and 37 °C, none *V. harveyi* cfu were detected in all the experiments (Fig. 2).

3.2. Evaluation Acanthamoeba castellanii Neff movement

After 6 days *A. castellanii* trophozoites had completed the total distance of 5,91 cm and could reach the *V. harveyi* inoculum (Fig. 3). Nevertheless, the amoeba from the negative control plates needed almost 10 days to migrate to the other petri plate side (data not shown).

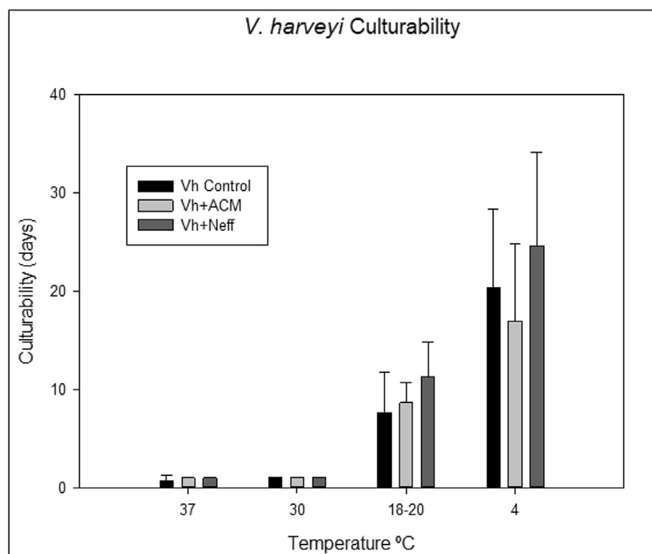

Fig. 2. Culturability assay at 4 different temperatures growing weakly. Vh Control: 1 ml *V. harveyi* 10^7 cfu/ml + 1 ml PYG supplemented medium; Vh+ACM: *V. harveyi* 10^7 cfu/ml + 1 ml ACM; Vh + Neff: *V. harveyi* 10^7 cfu/ml + 1 ml *Acanthamoeba castellanii* Neff 10^6 amoeba/ml. Error bars show standard deviations.

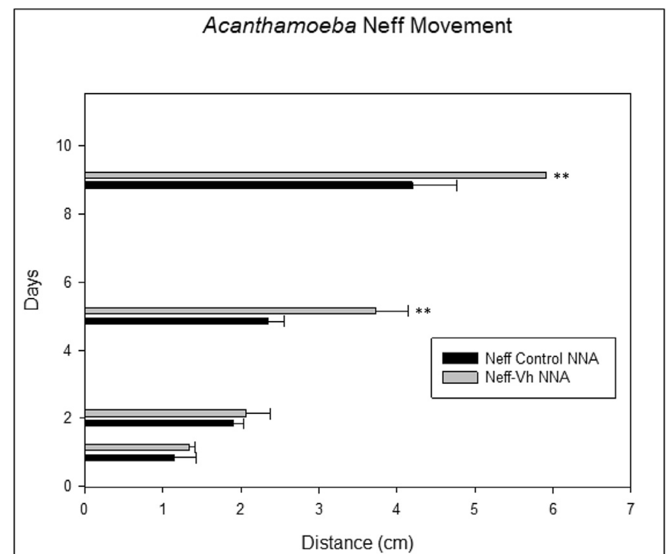

Fig. 3. *Acanthamoeba castellanii* Neff migration measurement. Neff control NNA: *A. catellani* Neff in NNA plates with no *V. harveyi* inoculum. Neff-Vh: *A. catellani* Neff in NNA plates in presence of *V. harveyi* inoculum. Error bars show standard deviations. One Way ANOVA, P<0.050.

4. Discussion

Nowadays, a high number of studies are based on the evaluation of the relation between bacteria species and FLA species, such as *Legionella pneumophila* (Fouque et al., 2014; Thomas et al., 2010, 2008), *Mycobacterium chelonae* (Cabello-Vílchez et al., 2014), *Campylobacter jejuni* (Vieira et al., 2015) or *Vibrio cholera* (Shanan et al., 2016). Some bacteria species are capable to avoid amoeba digestion and they could resist to the hostile external conditions. There are some bacteria species which are able to proliferate and leave amoeba environment when the external conditions will be favorable again (Strassmann and Shu, 2017; Balczun and Scheid, 2017; Guimaraes et al., 2016; Scheid, 2014; Casadevall, 2008; Hilbi et al., 2007; Axelsson et al., 2005; Greub and Raoult, 2004).

In the Canary Islands (Spain) have been reported almost 20 Vibrio species from 1936 to the present, within which we can find *V. alginolyticus*, *V. harveyi* and *V. chagasii* among others (OAG, 2011). Specifically, *Vibrio harveyi* is known as the most abundant isolated marine Vibrio and is able to infect the damaged tissues frequently ending up in fish death, producing dramatic economic losses for the aquaculture industry (Harikrishnan et al., 2011). One of the most important virulence characteristics of *V. harveyi* is the ability to form biofilms, related to the extracellular self-secreted exopolysaccharides (EPS) matrix. As it has been mentioned above, biofilm formation contribute to the establishment of FLA cultures in the environment (Preston et al., 2001; Khan, 2006; Scheid et al., 2008) and, on the other hand, FLA could act as a protective host for some bacteria species. This is a both side relation which highlights the importance of studying bacteria-amoeba interactions.

This is the first time that *V. harveyi* and *A. castellanii* Neff interaction has been studied. Within FLAs related to human infections group, *Acanthamoeba* spp. is the most abundant amoeba in the environment and can be easily isolated (Guimaraes et al., 2016). If we also talk about the prevalence in water bodies, *Acanthamoeba* spp. along *Vermamoeba* spp. are the most common genera described in the literature (Fouque et al., 2015; Richards et al., 2013; Lorenzo-Morales et al., 2007, 2015). For these reasons we have

decided to use this genus and, to be able to compare our results with other *Acanthamoeba*-bacteria interaction studies, we have chosen *Acanthamoeba castellanii* Neff. Despite the culturability of *V. harveyi* in presence of *A. castellanii* Neff has not presented significant differences, we have reported significant differences in *A. castellanii* Neff motility when is cultivated in presence of *V. harveyi* inoculum. In any case, it is necessary to distinguish between chemokinesis and chemotaxis in future studies. The first one occurs when the cell migration is stimulated by a symmetrically or asymmetrically applied factor without determining its direction. On the contrary, chemotaxis occurs when a soluble factor is applied asymmetrically and dictates the direction of cell migration (Petrie et al., 2009). For further assays it is important to evaluate which substances or molecules are the responsible of this possible chemoattraction, as it is also necessary make the same experiments in different amoebae strains.

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