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Natural genetic transformation of *Vibrio parahaemolyticus* via pVA1 plasmid acquisition as a potential mechanism causing AHPND

Gerardo de Jesús Carrillo-Méndez[1], Lina Angélica Zermeño-Cervantes[2], Alberto Antony Venancio-Landeros[3,5], Sergio Francisco Martínez Díaz[2], César Salvador Cardona-Félix[4,*]

[1]Departamento Académico de Biología Marina, Universidad Autónoma de Baja California Sur, 23080 La Paz, BCS, Mexico
[2]Instituto Politécnico Nacional-CICIMAR, 23096 La Paz, BCS, Mexico
[3]Instituto de Biotecnología, Universidad Nacional Autónoma de México, 62210 Cuernavaca, Mor., Mexico
[4]CONACyT-Instituto Politécnico Nacional-CICIMAR, 23096 La Paz, BCS, Mexico

[5]*Present address:* Gene2Life, 36615 Irapuato, Gto., Mexico

ABSTRACT: *Vibrio parahaemolyticus* is the causative bacterium of acute hepatopancreatic necrosis disease (AHPND) in white shrimp *Litopenaeus vannamei*. This bacterium secretes protein toxins whose genes are encoded in an auto-transmissible plasmid called pVA1. The presence of this plasmid in *V. parahaemolyticus* is determinant for disease development. Its propagation is not only linked to bacterial colonisation capacity but also to horizontal gene transfer mechanisms. Nevertheless, the active uptake of plasmid, which is known as natural genetic transformation (NGT), has not yet been proposed as a possible acquisition mechanism of the pVA1 plasmid among *Vibrio* species. Previous studies suggest that some *Vibrio* species have the ability to undergo NGT in the presence of chitin. Therefore, the objective of this study was to evaluate the induction of NGT mediated by chitin in *V. parahaemolyticus* (ATCC-17802) through its ability to incorporate and express the pVA1 plasmid. The results showed that a reference strain that does not initially contain the plasmid can incorporate the plasmid under the appropriate transformation conditions, and cause mortality in white shrimp similar to that observed for pathogenic strains isolated from infectious outbreaks. Given the management and conditions of a shrimp farm with large amounts of chitinous exoskeletons, it is feasible that NGT could be a possible acquisition mechanism of plasmid pVA1 among *Vibrio* species, turning a non-causative strain of *V. parahaemolyticus* into a causative strain. With this study, we have expanded the knowledge of the pathogenesis process mediated by NGT and the understanding of the possible propagation mechanisms of emerging diseases in the aquaculture sector.

KEY WORDS: Virulence factors · *Vibrio parahaemolyticus* · Acute hepatopancreatic necrosis disease · AHPND · Natural genetic transformation

1. INTRODUCTION

Natural genetic transformation (NGT) is the active uptake of free DNA by bacterial cells for heritable incorporation (Håvarstein 1998, Chen & Dubnau 2004, Sun et al. 2013), and represents one of the main mechanisms of horizontal gene transfer in bacteria (Lorenz & Wackernagel 1994). This phenomenon has been shown to occur naturally in the environment, given the ability of bacteria to propagate their genetic information and acquire new genotypic and phenotypic characteristics that favour their performance in

*Corresponding author: cscardonafe@conacyt.mx

different environments (Lorenz & Wackernagel 1994, Sun et al. 2013).

In the family *Vibrionaceae*, the genus *Vibrio* is composed of 14 recognised clades (Sawabe et al. 2013). Several of them are important etiological disease agents in both humans and marine organisms (McCarter 1999). In particular, *V. parahaemolyticus* has been recognised as being responsible for infectious outbreaks in shrimp cultures from the order Penaeidae (*Litopenaeus vannamei* and *Penaeus monodon*) (Lightner et al. 2012, Chamberlain 2013, Zorriehzahra & Banaederakhshan 2015). The production of *L. vannamei* has been affected by acute hepatopancreatic necrosis disease (AHPND) (Tran et al. 2013), which has had a severe impact on the shrimp industry, causing losses worth millions of US dollars and reducing the competitiveness of this industry worldwide (Zorriehzahra & Banaederakhshan 2015).

Strains of *V. parahaemolyticus* associated with AHPND (Vp_{AHPND}) are characterised by the production of a harmful binary toxin (called PirAB) that affects the shrimp hepatopancreas. This toxin is encoded by the plasmid pVA1. Plasmid pVA1 also contains genes involved in horizontal gene transfer, so it is considered a self-transmissible plasmid (Lee et al. 2015).

The genus *Vibrio* is susceptible to experimental NGT (Frischer et al. 1990); factors such as temperature, nutrient availability and presence of adhesion substrates greatly affect this characteristic (Frischer et al. 1993, Meibom et al. 2005). Particularly in *V. cholerae*, metabolites that derive from chitin, such as *N*-acetylglucosamine (GlcNAc) (Payne-Delíz et al. 2015), are important inducers of the expression of genes involved in cellular structure formation, which capture and incorporate extracellular genetic material (Meibom et al. 2005, Shime-Hattori et al. 2006).

Chitin is considered to be the second most abundant natural polymer on the planet, after cellulose (Hudson & Smith 1998). It is a cell wall component in filamentous fungi and an important part of crustacean and insect exoskeletons. Therefore, it is considered an essential structural biomolecule (Winkler & Kaplan 2001).

NGT in *Vibrio* can occur between closely related species, such as *V. cholerae* and *V. mimicus* (clade Cholerae), or *V. harveyi* and *V. campbellii* (clade Harveyi) (Sawabe et al. 2007). However, recently, the pVA1 plasmid has been found in a *Vibrio* species from the Orientalis clade (*V. punensis* sp. nov. strain BA55) (Restrepo et al. 2018), which was not considered pathogenic for crustaceans (Hada et al. 1984, Gomez-Gil et al. 2008). This finding shows that the ability of *Vibrio* to acquire genes by different species goes beyond the limits between clades.

Currently, extensive knowledge of NGT processes is available in different bacterial species, but studies conducted on *V. parahaemolyticus* are scarce, and most of them only cover an exploratory context where hypothetical scenarios of competitive induction in natural environments are discussed.

Therefore, the aim of this study was to induce NGT through the use of chitin in *V. parahaemolyticus* reference strain ATCC-17802.

2. MATERIALS AND METHODS

2.1. Bacterial strains

The Vp_{AHPND} strain donor of the pVA1 plasmid was isolated from a shrimp farm in Sinaloa, Mexico (hereinafter: M9_Vp_{AHPND}+) (Zermeño-Cervantes et al. 2018). The *Vibrio parahaemolyticus* reference strain was obtained from the American Type Culture Collection (ATCC-17802) (hereinafter: Vp_{17802}) and used as a pVA1 plasmid receptor. Verification of purity and morphological characteristics of the strains was performed by Gram staining and culturing in selective (thiosulphate–citrate–bile salts–sucrose [TCBS] agar and CHROMagar™ Vibrio) and non-selective (marine agar and blood agar) media.

2.2. Induction of Vp_{17802} transformation

A genetic transformation protocol for *V. parahaemolyticus* was established based on studies by Shime-Hattori et al. (2006), Chen et al. (2010) and Gode-Potratz & McCarter (2011). Cell biomass of 50 ml from a 2 h culture of Vp_{17802} in marine broth (Bacto peptone 5 g, yeast extract 1 g, Fe_3SO_4 0.002 g l^{-1} seawater) was harvested by centrifugation (3925 × *g* for 20 min). Cells were resuspended in 50 ml of fresh marine broth; 0.4 g of powdered chitin (Merck), previously sterilised, was added to 5 ml of the bacterial suspension. The culture was incubated at 35°C for 24 h without agitation.

Separately, a 24 h culture of M9_Vp_{AHPND}+ in marine broth was inactivated through water bath heating at 90°C for 10 min. After clarifying the culture by centrifugation (15890 × *g* for 20 min) and filtering through a 0.22 µm filter, 300 µl were taken and added to the tube with chitin and the Vp_{17802} strain. The mixture was incubated without agitation for 24 h at 30°C. Finally, a chitin-absent culture was included as control.

Cells from both treatments were resuspended with vigorous shaking and spread on marine agar using the cross-streak method.

2.3. Verification of Vp_{17802} strain transformation

The transformation confirmation of Vp_{17802} with the genetic material of M9_Vp_{AHPND}+ (which includes the plasmid pVA1) was performed using a colony PCR protocol and multiplex PCR of 3 fragments. The first amplification product comprised the gene that encodes one of the toxins responsible for hepatopancreatic damage in shrimp (GenBank accession no. NC_025152.1), the so-called Id_PirA (333 bp) (forward primer: 5′-ATG AGT AAC AAT ATA AAA CAT GAA AC-3′; reverse primer: 5′-GTG GTA ATA GAT TGT ACA GAA-3′) (Sirikharin et al. 2015). The second fragment corresponds to a unique region from chromosome II in *V. parahaemolyticus* (GenBank KP324996.1), so-called Id_Vp (1000 bp) (forward primer: 5′-ACC TTG ATG GAA GAT AAC GCC C-3′; reverse primer: 5′-ATC AGC GGC GCA TCC ATA CA-3′). Finally, a fragment was amplified from the pVA1 plasmid (GenBank KP324996.1), so-called Id_pVA1 (558 bp) (forward primer: 5′-GTC TCT GCA CTC GAA AGG CAC TTA G-3′; reverse primer: 5′-GAG TGG GTT TGT GCT CAA GTG CC-3′).

The selection of marker genes used was performed by sequence analysis of pVA1 plasmid and chromosomes I and II from *V. parahaemolyticus*, avoiding the selection of redundant sequences. PCR temperature and conditions were similar to those described by Sirikharin et al. (2015) for the amplification of the AP3 gene (that codes for the PirA toxin). PCR amplification products were visualised on a 1% agarose gel. The positive Vp_{17802} colonies for AP3 gene and plasmid pVA1 (hereinafter: $Vp_{17802+pVA1}$) were spread on marine agar by cross streak, as were the Vp_{17802} and M9_Vp_{AHPND}+ strains, for visualising colonial morphology.

2.4. *Litopenaeus vannamei* challenge test and histology of hepatopancreatic tubule tissue

Increase in Vp_{17802} virulence due to the incorporation of pVA1 plasmid (strain $Vp_{17802+pVA1}$) was evaluated in a bioassay with *L. vannamei* and visualisation of hepatopancreatic tubules. An experimental design was performed consisting of 3 treatments and 1 blank, in quadruplicate: Treatment 1: M9_Vp_{AHPND}+ strain (positive control); Treatment 2: Vp_{17802} strain (negative control); Treatment 3: $Vp_{17802+pVA1}$ strain; and Treatment 4: blank without treatment. Experimental 4 l containers with natural seawater of salinity 35‰ were used, previously filtered and sterilised, with 10 shrimp (~3 g each) per experimental unit. This experiment was kept at a controlled temperature of 30°C and constant aeration.

To reproduce AHPND, an acute infection was induced according to the World Organisation for Animal Health manual (OIE 2018), with a modification made through the administration of the pathogen in feeding.

Commercial pulverised feed (30%) mixed with bacterial biomass (70%) of the 3 strains (each prepared separately) was supplied equal to 5% of total shrimp biomass per experimental unit. Shrimp survival was monitored every hour for 24 h. One of the experimental units from each treatment was designated to provide sample organisms for fresh analysis of the hepatopancreas according to the methodology established by Cuéllar-Anjel (2014). Samples for histology were fixed in Davidson's fixative and 5–7 µm thick sections of cephalothorax tissue were stained with haematoxylin and eosin (H&E) to reveal typical manifestations of AHPND pathology (Tran et al. 2013).

Normality (Kolmogorov-Smirnov) and homoscedasticity (Levene) of obtained data were confirmed. Subsequently, a 1-way ANOVA and Tukey's multiple comparisons test were performed with R statistical software version 3.5.1 (R Foundation for Statistical Computing).

3. RESULTS

Growth of the $Vp_{17802+pVA1}$ strain showed morphological differences with respect to the non-transformed strain Vp_{17802} after chitin treatment. Its ability to move on the agar surface increased and colony thickness decreased (Fig. 1). The Vp_{17802} transformation to $Vp_{17802+pVA1}$ in the presence of chitin was confirmed through multiplex PCR (Fig. 2, lanes 10–13) by amplifying 3 fragments, which were similar to the positive control (M9_Vp_{AHPND}+; Fig. 2, lanes 6–9). Amplification of these fragments confirmed the correct transformation of the Vp_{17802} strain with the pVA1 plasmid, as well as the presence of the gene encoding the PirA toxin.

It is important to mention that in the treatment without chitin, such an amplification pattern was not observed (Fig. 2, lanes 2 and 3), suggesting that in that treatment, the Vp_{17802} strain was unable to acquire the genetic material of the donor strain.

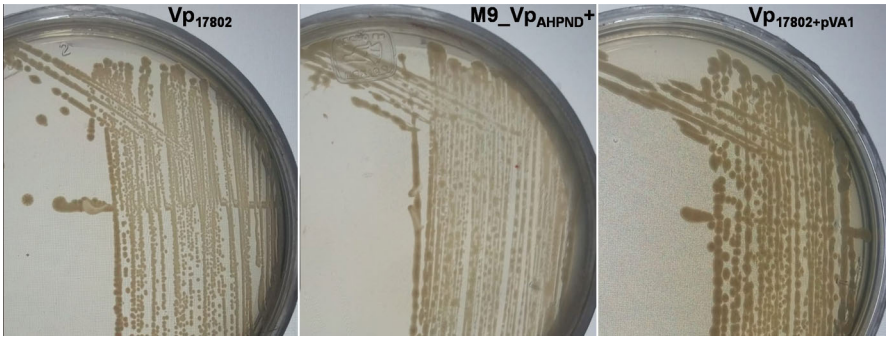

Fig. 1. Colonial morphology of the Vp_{17802} (non-transformed), M9_Vp_{AHPND}+ (pVA1 plasmid carrier and donor), and $Vp_{17802+pVA1}$ (transformed with pVA1 plasmid) strains of *Vibrio parahaemolyticus*

To demonstrate the acquisition of attributes related to the virulence conferred by the pVA1 plasmid of the M9_Vp_{AHPND}+ strain, a bioassay was performed using white shrimp *Litopenaeus vannamei*. A rapid decrease in shrimp survival (to 20%) was observed during the first hour post-treatment (Fig. 3) in experimental containers with the $Vp_{17802+pVA1}$ and M9_Vp_{AHPND}+ treatments. On the other hand, 100% survival was maintained in the treatments with the reference strain Vp_{17802} and the blank control. Survival in the second hour with the Vp_{17802} treatment decreased to 93.3%, showing significant differences ($p < 0.001$) with respect to the $Vp_{17802+pVA1}$ and M9_Vp_{AHPND}+ treatments; in the latter 2 groups, survival fell to zero in the second hour, simultaneously and statistically equally between them ($p > 0.05$). Finally, the blank control group maintained 100% survival throughout the entire experiment.

Analysis of the hepatopancreatic tubules from shrimp treated with $Vp_{17802+pVA1}$ (Fig. 4A) and M9_Vp_{AHPND}+ (Fig. 4B) demonstrated dysfunction of the hepatopancreas caused by progressive lesions of the epithelial cells, whereas these injuries were not observed in shrimp treated with the reference strain Vp_{17802} (Fig. 4C), nor in the tubules of untreated shrimp (Fig. 4D). The histopathological analysis showed a sloughing of the hepatopancreatic epithelial cells in the shrimps challenged with strain $Vp_{17802+pVA1}$ (Fig. 5A) and M9_Vp_{AHPND}+ (positive control) (Fig. 5B), characteristic of AHPND. The hepatopancreatic tissue of shrimps challenged with Vp_{17802} (negative control) lacked these signs and was similar to that of the non-challenged shrimps (blank control), with the hepatopancreatic tubules coated with epithelial cells and other specialised cells (Fig. 5C,D).

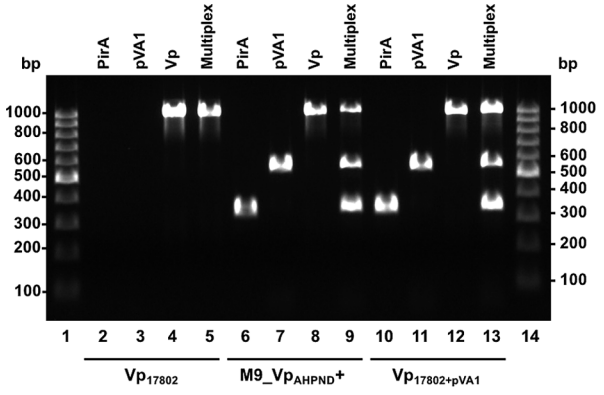

Fig. 2. PCR of 3 fragments (see Section 2.3) of the *Vibrio parahaemolyticus* strains. Lanes 1 and 14: molecular weight marker. Lanes 2–5 (negative control, Vp_{17802}): absence of Id_PirA and Id_pVA1, presence of Id_Vp, and multiplex PCR. Lanes 6–9 (positive control, M9_Vp_{AHPND}+): presence of Id_PirA (333 bp), Id_pVA1 (558 bp), and Id_Vp (1000 bp); and multiplex PCR. Lanes 10–13 ($Vp_{17802+pVA1}$): presence of Id_PirA, Id_pVA1 PCR, and Id_Vp; and multiplex PCR

4. DISCUSSION

PirAB was described in 2013 as a toxin produced by *Vibrio parahaemolyticus* strains. It has been identified as being responsible for AHPND in

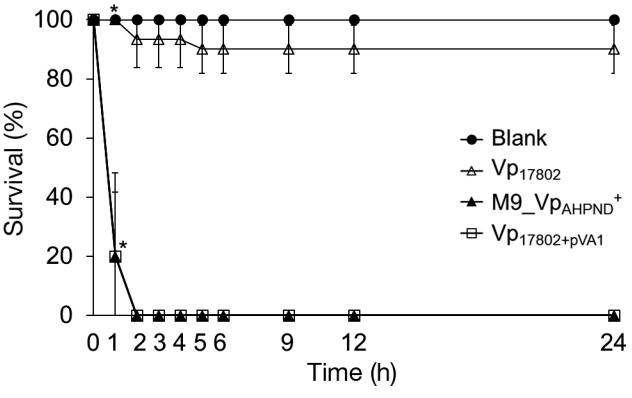

Fig. 3. Survival of *Litopenaeus vannamei* challenged with the *Vibrio parahaemolyticus* strains: $Vp_{17802+pVA1}$, M9_Vp_{AHPND}+ (positive control), Vp_{17802} (negative control), and no treatment (blank control). Data are means ± SD. *Significant difference ($p < 0.001$)

penaeid shrimps recorded since 2009 (Tran et al. 2013). Versatility in the propagation of the genes encoding these toxins through horizontal gene transfer (conjugation, transposition or homologous recombination) (Lee et al. 2015, Hong et al. 2016, Lo et al. 2016) has been described as being the mechanisms responsible for its dissemination between *V. parahaemolyticus* strains and other *Vibrio* species such as *V. harveyi*, *V. owensii* and *V. campbellii* (Dong et al. 2017, Xiao et al. 2017, Liu et al. 2018). Nevertheless, NGT has not been proposed until now as a possible acquisition mechanism of the pVA1 plasmid among *Vibrio* species.

The experimental conditions of the present study through chitin induction demonstrated the competence and transformation phenomenon in *V. parahaemolyticus* (Vp_{17802}) with the pVA1 plasmid of a donor strain identified as positive for AHPND (Fig. 2). This phenomenon of natural competence through chitin interaction has been widely documented in *V. cholerae* (Bartlett & Azam 2005, Meibom et al. 2005, Seitz & Blokesch 2013, Antonova & Hammer 2015). Among other benefits, binding to chitinous exoskeletons from shrimp provides a nutrient-rich habitat to *V. parahaemolyticus* (Heidelberg et al. 2002), and increases both its survival during temperature stress (Amako et al. 1987) and protein expression for binding and colonisation involved in pathogenesis (Kirn et al. 2005, Reguera & Kolter 2005). Altering its physiology to express the related machinery for inducing natural competence to incorporate extracellular DNA can give it new attributes (Meibom et al. 2005).

The transformation effects were also observed through the reduction of *Litopenaeus vannamei* survival caused by $Vp_{17802+pVA1}$ (Fig. 3), the hepatopancreatic tubular constriction (Fig. 4) and by the sloughing of hepatopancreatic epithelial cells in the shrimps challenged with $Vp_{17802+pVA1}$ (Fig. 5), which demonstrates that incorporation of the pVA1 plasmid induced by chitin conferred a new attribute to the reference strain Vp_{17802} in its degree of virulence. The results agree

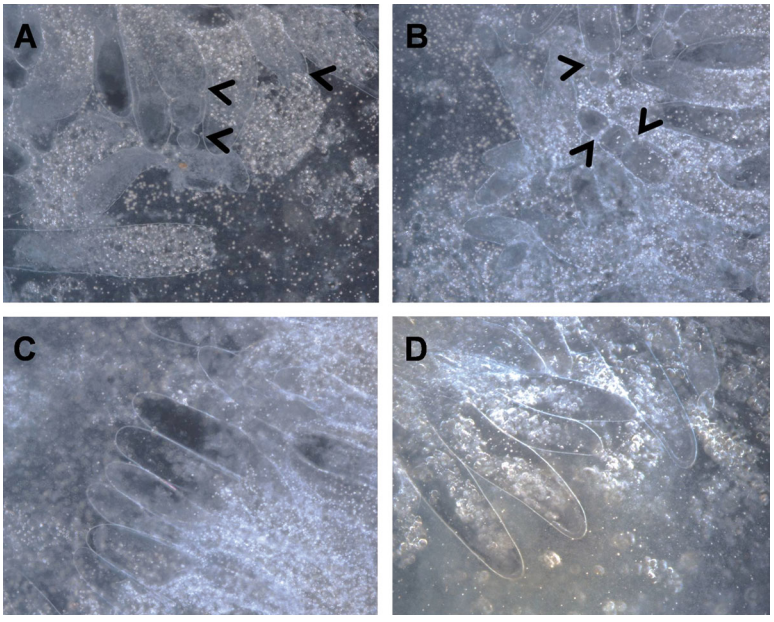

Fig. 4. Histology of hepatopancreatic tubules from *Litopenaeus vannamei* challenged with *Vibrio parahaemolyticus* strains. Hepatopancreatic tubular constriction (arrows) with (A) $Vp_{17802+pVA1}$ and (B) M9_Vp_{AHPND}+ (positive control). Healthy tubules with (C) Vp_{17802} (negative control) and (D) no treatment (blank control)

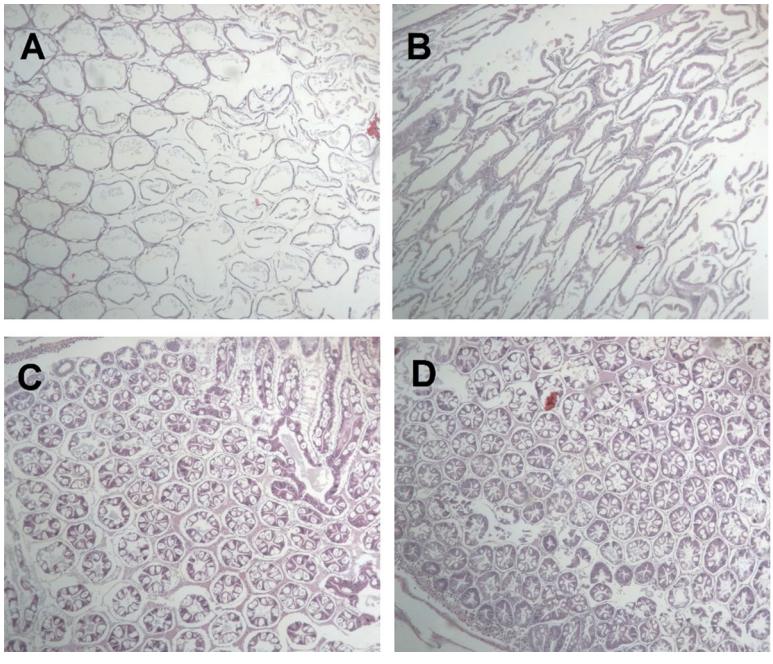

Fig. 5. Cross-section of H&E-stained hepatopancreatic tissue from *Litopenaeus vannamei* challenged with *Vibrio parahaemolyticus* strains. Sloughed cells and necrotic hepatopancreatic tubule epithelia, characteristic of acute hepatopancreatic necrosis disease (AHPND), with (A) $Vp_{17802+pVA1}$ and (B) M9_Vp_{AHPND}+ (positive control). Normal histology, with the lumens enclosed by epithelial cell layers that comprise specialised cells, with (C) Vp_{17802} (negative control) and (D) no treatment (blank control)

with those reported by Lee et al. (2015), who demonstrated that the AHPND-causing strains of *V. parahaemolyticus* contain the plasmid pVA1 with a post-segregational killing system and that the ability to cause disease is abolished by the natural absence (in the present study, the reference strain Vp_{17802}) or experimental deletion of the plasmid-encoded toxins PirA and PirB. Consequently, a non-virulent strain becomes capable of causing AHPND upon acquisition of this plasmid, as demonstrated with $Vp_{17802+pVA1}$.

It is worth mentioning that an acute infection was induced by administering large quantities of pathogen through food, since managing and maintaining the organisms under controlled conditions made it difficult to reproduce the disease experimentally.

Although natural competence is well described in *V. cholerae*, most studies have been conducted with reference strains (A1552, V060002, C6706) (Meibom et al. 2005, Yamamoto et al. 2010, Antonova & Hammer 2011) and only 1 wild strain (E7946) (Dalia et al. 2014). It is still unknown how common this natural competence event is among *V. cholerae* strains from different sources (Antonova & Hammer 2015).

Natural transformation has been demonstrated in other *Vibrio* species, such as *V. parahaemolyticus*, *V. vulnificus* and *V. fischeri* (Gulig et al. 2009, Chen et al. 2010, Pollack-Berti et al. 2010). Considering that the chitin degradation pathway (production of chitinolytic enzymes) is a conserved function among the members of this genus (Hunt et al. 2008), interaction with this substrate could induce natural competence in other species in the same manner.

Shrimp intensive-culture conditions have favoured natural competence in *Vibrio*, a phenomenon that occurs more frequently in natural conditions, which is due to the high incidence of *Vibrio* in culture systems and their multiplication and persistence in a medium with high concentrations of organic matter (Austin 2012). Similarly, a triggering factor for NGT is the accumulation of chitin exoskeletons in culture ponds (Kungvankij et al. 1986, Weis et al. 1992).

Under natural conditions, the concentration of extracellular DNA has been estimated in the range of tens of micrograms per litre of water, while it is approximately 3 orders of magnitude higher or more within biofilms in sediments (Whitchurch et al. 2002, Corinaldesi et al. 2005).

In culture conditions, *Vibrio* growing on the exoskeleton surface (of organisms or moults) has good and sufficient conditions to become competent and also has available high concentrations of exogenous DNA for its transformation.

5. CONCLUSION

The success of the transformation was established by the ability of the ATCC-17802 strain to incorporate pVA1 plasmid from a wild strain Vp_{AHPND}+. Success in the experimental transformation of a *Vibrio parahaemolyticus* strain that does not initially contain the pVA1 plasmid, using chitin interaction, and demonstration of its increasing virulence, suggests that under culture conditions, the presence of chitinous exoskeletons could favour the acquisition of the pVA1 plasmid among strains of *Vibrio* through NGT, along with other documented horizontal gene transfer mechanisms. With these results, knowledge of the pathogenesis process, mediated by NGT, could expand and improve understanding of the propagation mechanisms of emerging diseases, with emphasis on the aquaculture sector.

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