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Vibrio harveyi Adheres to and Penetrates Tissues of the European Abalone Haliotis tuberculata within the First Hours of Contact

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Vibrio harveyi is a marine bacterial pathogen responsible for episodic epidemics generally associated with massive mortalities in many marine organisms, including the European abalone Haliotis tuberculata. The aim of this study was to identify the portal of entry and the dynamics of infection of V. harveyi in the European abalone. The results indicate that the duration of contact between V. harveyi and the European abalone influences the mortality rate and precocity. Immediately after contact, the epithelial and mucosal area situated between the gills and the hypobranchial gland was colonized by V. harveyi. Real-time PCR analyses and culture quantification of a green fluorescent protein-tagged strain of V. harveyi in abalone tissues revealed a high density of bacteria adhering to and then penetrating the whole gill-hypobranchial gland tissue after 1 h of contact. V. harveyi was also detected in the hemolymph of a significant number of European abalones after 3 h of contact. In conclusion, this article shows that a TaqMan real-time PCR assay is a powerful and useful technique for the detection of a marine pathogen such as V. harveyi in mollusk tissue and for the study of its infection dynamics. Thus, we have revealed that the adhesion and then the penetration of V. harveyi in European abalone organs begin in the first hours of contact. We also hypothesize that the portal of entry of V. harveyi in the European abalone is the area situated between the gills and the hypobranchial gland.

The halophilic Gram-negative bacterium Vibrio harveyi is a common pathogen of many marine vertebrate and invertebrate species (1). V. harveyi is known to induce gastroenteritis, inflammation of the circulatory system or eye, and skin lesions in various species of fishes, crustaceans, and mollusks (2). In the European abalone Haliotis tuberculata, V. harveyi is responsible for the massive mortalities that occur during the summer spawning period both in natural populations and in farmed stocks (3, 4). Vibriosis of the European abalone is characterized by the presence of white spots on the foot and inflammation of the pericardial tissue, resulting in impaired mobility and septicaemia (5).

Schematically, the bacterial infection process can be summarized to occur in three stages: (i) colonization, adhesion of the pathogen to the host surface, and initial multiplication and then penetration of the pathogen into the body through one or several portals, (ii) invasion of the host organs and/or the circulatory system concurrently with the expression of virulence factors by the pathogen, and (iii) exit of the pathogen and transmission of the disease (6, 7). Invasion and multiplication of V. harveyi inside the circulatory system, the hemolymph, seem to take place after 24 h of contact with the European abalone (5), suggesting that the adhesion and penetration of V. harveyi must occur in the first 24 h of contact. The portal of entry and the precise timing of the earlier stages of V. harveyi infection in the European abalone remain uncharacterized.

Many studies which have focused on the infection dynamics of fish pathogens have revealed a wide variety of modes of infection among Vibrio species. For example, V. anguillarum seems to penetrate preferentially through the gastrointestinal tract in the turbot Scophthalmus maximus (8), while both the skin and gills are the portal of entry in the Atlantic salmon (Salmo salar) (9) and in the rainbow trout (Salmo gairdneri) (10). In marine mollusks, the organic matrix of the shell may be a putative portal of entry of Vibrio pathogen, as observed with V. tapetis, which is known to invade Ruditapes philippinarum clams after adhesion on the periostracal lamina shell and the extrapallial fluid (11, 12). Considering the different modes of infection in pathogenic Vibrio species, the objectives of this study were to localize and verify the existence of a portal of entry of V. harveyi in the European abalone and to determine the infection dynamics occurring during the early stages of vibriosis.

MATERIALS AND METHODS

Abalones and bacterial strains. Sexually mature abalones were transported in containers filled with macroalgae (Palmaria palmata) from the France Haliotis Hatchery, Plouguerneau, France, to our laboratory at the European Institute for Marine Studies, Brest, France, in June 2011. The abalones were used in the experiments if they did not present any tissue injuries or pedal muscle pustules and were capable of moving and adhering to the tank surface. The abalones were placed in 5-liter tanks of seawater, which was replaced daily with seawater pumped from the Bay of Brest (dissolved oxygen concentration, >7.5 mg _l^-1_; salinity, 35‰) and filtered through a 1-μm-pore-size filter. The seawater temperature was increased by 1°C per day until it reached 19°C, and the animals were then maintained at this temperature for 1 week before beginning the experiments.

Two virulent strains of V. harveyi were used in this study. The first strain, ORM4, had been isolated from diseased H. tuberculata abalones in Normandy, France, during an episode of massive mortalities in 1999 and was affiliated with V. carchariae, the synonym species of V. harveyi, after...
16S rRNA gene sequencing and phylogenetic analysis (3, 13). The second strain was a green fluorescent protein (GFP)-tagged and kanamycin-resistant bacterium constructed from the ORM4 strain by Travers et al. (5). The initial ORM4 strain and the GFP-tagged ORM4 strain have similar growth rates and virulences, and the GFP-labeled plasmid is stable in the GFP-tagged ORM4 strain for 8 days (5). Bacteria were grown in Luria-Bertani broth (LB; Sigma) supplemented with salt (LBS; final concentration, 20 g·liter$^{-1}$) in a temperature-controlled shaker at 28°C for 16 h before challenge. Prior to use in experiments, bacterial cultures were washed and resuspended in sterile seawater.

**Experiment 1: effect of contact duration on abalone mortality.** Five triplicate groups of 15 sexually mature abalones ($n = 270$) were placed in tanks in seawater. One hundred microliters of the diluted tissue homogenates was plated onto LBS agar plates and treated with 100 µg·ml$^{-1}$ kanamycin, to ensure the selection of GFP-tagged bacteria. The dishes were incubated overnight at 28°C, and the number of colonies fluorescing under UV light was counted. The number of colonies per 100 µg of tissue (density) or 1 ml of hemolymph (concentration) is reported.

**Bacterial quantification by real-time quantitative PCR.** (a) DNA extraction procedure. DNA extraction was done on hemolymph and untreated tissue samples. Five hundred microliters of hemolymph or homogenates of untreated tissue were diluted in 500 µl of extract salted buffer (100 mM Tris HCl, 100 mM NaCl, 50 mM EDTA, pH 8). Total DNA was extracted using a traditional phenol-chloroform-isomyl alcohol method (13), after treatment with RNAse A (10 µg/ml), lysozyme (10 mg/ml), 10% (vol/vol) SDS, 10% (vol/vol) Sarkosyl, and protease K (25 mg/ml). Finally, DNA was suspended in 50 µl of DNAse-free water. The concentration and quality of the nucleic acids were determined with a spectrometer (NanoDrop ND-1000; Thermo Fisher Scientific, USA).

The standard samples used for qPCR were obtained after DNA extraction from 500 µl of hemolymph or 100-µg homogenates of organs dissected from noninfected abalones and supplemented with 10-fold serial dilutions of *V. harveyi* from 10$^0$ to 0 CFU. Viable bacteria were counted by plating and optical microscopy.

(b) Real-time quantitative PCR conditions and analysis. The concentration of *V. harveyi* in hemolymph was measured by qPCR using a 7500 Fast real-time PCR system and TaqMan Universal master mix (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA). Amplification was done with *V. harveyi* toxR gene-specific primers: forward primer CCA-CTG-CTG-AGA-CAA-AAG-CA and reverse primer GTG-ATT-CTG-CAG-GGT-TGG-CTG-AGC-GAC-GTG-CCG-AAC-AAG-GT-CG and TaqMan probe concentrations, each reaction was run in duplicate with a final volume of 20 µl containing 4 µl of DNA sample, a 1X concentration of TaqMan master mix, 300 nM each primer, and 200 nM probe. The reactions were initiated by activation of the Thermo-Start DNA polymerase for 10 min at 95°C, followed by 45 amplification cycles (denaturation at 95°C for 15 s, annealing at 60°C for 1 min). The threshold cycle ($C_T$) was defined as the first PCR cycle in which the probe reporter fluorescence was detectable above a baseline signal and was inversely proportional to the logarithm of the initial bacterial density or concentration. Thus, $C_T$ values, obtained for each sample using 7500 software (v2.0.3), were compared with those on standard curves to determine the initial *V. harveyi* density or concentration.

The sensitivity of the amplification was estimated for each abalone organ from three separate PCR assays. The primer efficiency ($E$) was determined from the slope of the standard curves using the method described by Yuan et al.: $E = 10^{1/(C_T - C_{T0})}$ (17).

The influence of time on bacterial density or concentration was assessed for the different organs using a nonparametric Wilcoxon test. Differences in bacterial concentrations or densities between two infection intervals were detected with a Wilcoxon signed-rank test (18). Statistical tests were done using JMP (v9.0.0.0) software (SAS Institute Inc.).

**RESULTS**

**Effect of contact duration on abalone mortality.** European abalone mortality due to vibriosis was assessed over a 12-day period after different durations of contact with *V. harveyi* (Fig. 1; Table 1). The cause of mortality was assessed each day by detection of *V. harveyi* in abalone hemolymph. The cumulative mortality rate of infected abalones increased, while the lethal time period (LT$_{50}$), which was the time period over which 50% of the abalones died, decreased throughout the different contact durations (Table 1). Kaplan-Meier analyses inferred that mortality curves were different between 1 h and 6 h of contact ($P = 0.0114$) and between 6 h
and 24 h of contact (P = 0.042). Contact with V. harveyi for 1 h was sufficient to induce 80% and 77% mortality in two tanks, whereas only one abalone died in the last one after 11 days of the experiment. Abalone mortality reached 72% and 80% when abalones were in contact with V. harveyi for 6 h and 24 h, respectively, after 8 days of the experiment. No mortality was reported in the control groups.

Portal of entry. (i) Microscopic observations. fluorescent bacteria were observed on the surface of gill filaments immediately after contact with the GFP-tagged V. harveyi strain (data not shown). After 6 h of contact, fluorescent bacteria were aggregated on the epithelial and mucosal area between the gills and the hypobranchial gland (data not shown).

(ii) Quantification measures. Quantification by plating revealed that V. harveyi densities increased exponentially in all organs during the first few hours after contact and throughout the different contact times (Fig. 2). The density of the adhered GFP-tagged bacteria was the highest on the surface of the gills, being 5-fold greater than that in the digestive gland and 12-fold greater than that on the surface of the foot muscle after 6 h of contact (Fig. 2A). Penetrated bacteria were also 10-fold more numerous in the gills than in the digestive gland after 6 h of contact (Fig. 2B).

Efficiency and sensitivity of qPCR for V. harveyi quantification. The sensitivity and efficiency of V. harveyi qPCR quantification via toxR gene amplification were assessed with samples from the gills, digestive gland, foot muscle, and hemolymph (see Fig. S1 in the supplemental material). The sensitivity threshold was estimated to be 10^2 bacteria/100 mg of tissue for the gills, 10^3 bacteria/100 mg for the digestive gland, 10^3 bacteria/100 mg for the foot muscle, and 10^2 bacteria/ml for hemolymph. The equation of the regression line was then used to calculate the bacterial concentration or density from the qPCR data that were obtained. The average amplification efficiencies were 95% in the gills, 84% in the digestive gland, and 105% in the foot muscle and hemolymph.

Infection kinetics. The V. harveyi concentration depended on the contact duration in all abalone compartments (P < 0.01 by the Wilcoxon test). In hemolymph, the bacterial concentration increased with contact duration, except in samples in which the V. harveyi concentration was measured by the qPCR quantification method between 3 h or 6 h and 9 h of contact (Fig. 3; Table 2). In the gills, the density of adhered and penetrated vibrios also increased significantly with duration until 6 h of contact for both of the quantification methods used (Table 2). Moreover, the density of penetrated bacteria in the gills surpassed 10^3 CFU/100 mg after the first hour of contact, while that of the penetrated bacteria in the digestive gland and muscle did not reach the threshold of 10^2 CFU/100 mg until 6 h of contact (Fig. 3). For the digestive gland, quantification by plating revealed a significant increase in the density of either adhered or penetrated bacteria during infection. However, qPCR measures showed a stabilization of the V. harveyi densities in the digestive gland after 1 h, except that a difference was detected between 3 h and 6 h of contact (Fig. 3; Table 2). In the foot muscle, the V. harveyi density measured by plating increased significantly during the first hour and after 6 h of contact (Fig. 3; Table 2). Meanwhile, qPCR analysis of foot muscle tissue showed significant differences between controls and infected animals until 9 h of contact (Table 2).

For the majority of the measures, the bacterial count determined by qPCR analysis was slightly higher than the count determined by plating. No bacteria were counted either in samples
DISCUSSION

The duration of contact with the pathogen V. harveyi influenced the mortality rate and infection kinetics in European abalones (Fig. 1). Immediately after contact, a large number of bacteria could be observed in the gills and hypobranchial gland. Moreover, after 6 h of contact, the density of V. harveyi was at least 5-fold greater in the gills and in the hypobranchial gland than in the other tissues tested (Fig. 2). Finally, V. harveyi was detected in hemolymph after 3 h of contact (Fig. 3).

Our results show that the duration of contact between V. harveyi and the European abalones was correlated with the mortality rate. Moreover, 1 h of exposure was sufficient to induce mortality. This observation suggests that V. harveyi is able to adhere to and then penetrate abalone tissues during the first hour of contact. The relative rapidity of adhesion and penetration in the host has been described for many Vibrio species and for V. harveyi and may contribute to their symbiotic process. For example, in the association between the luminous bacterium V. fischeri and the sepia squid Euprymna scolopes, the symbiont is able to adhere to the ciliated epithelium of juvenile squid light organs after only 3 h (19). In the kuruma prawn Penaeus japonicus, the study of Vibrio sp. infection dynamics after oral inoculation showed that the pathogen can be detected in the stomach and hemolymph after 3 h and in all other sampled organs after 6 h (20). In the giant tiger prawn Penaeus monodon, modulation of immune gene expression obtained at t₀ or in samples from the controls by plating or by qPCR analysis.

**TABLE 2** Statistical significance of V. harveyi concentrations or densities in European abalone organs after the first hours of contact

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<sup>a</sup> Duration of contact between the European abalone and V. harveyi.

<sup>b</sup> HL, hemolymph.

<sup>c</sup> Differences were detected by the Wilcoxon signed-rank test (18). *, a statistically significant difference at P < 0.1; **, a statistically significant difference at P < 0.05; NS, not significant; ND, not detected.

FIG 3 Infection dynamics of Vibrio harveyi in European abalone tissues. Concentrations or densities (bacterial count per ml of hemolymph or per 100 mg of tissue) in hemolymph (A), gills (B), digestive gland (C), and foot muscle (D) were estimated during the first hours of contact. Results are given as the average concentrations or densities of five biological replicates, estimated by plating tissues from organs treated or not treated with ethanol on kanamycin-LBS agar dishes and by qPCR using specific primers and a TaqMan probe. Bars indicate standard errors. No bacterium was detected in samples obtained at t₀ or in control individuals immersed in filtered seawater without V. harveyi in suspension.
and of the synthesis of protein involved in immune function was observed in hemocytes after 6 h of immersion with a pathogenic strain of \( V.\) \( h\)arveyi, confirming its adhesion, penetration, and invasion capacities during the first hours of contact (21, 22).

The quantification of adhered or penetrated \( V.\) \( h\)arveyi by plating showed a high density of \( V.\) \( h\)arveyi on the surface and then within gill filaments and the hypobranchial gland after 1 h of contact. Microscopic observation confirmed a tropism of \( V.\) \( h\)arveyi for the epithelial and mucosal region situated between the gills and the hypobranchial gland in the European abalone. Previous studies showed that gills seem to be an ideal place of adhesion and, hence, are a relevant entry point for \( Vibrio\) pathogens in marine mollusks. In the case of the challenge with a GFP-tagged virulent strain of \( V.\) \( p\)araaeromonas, the density of the bacteria was maintained in the gills of \( Tiostrea\) \( c\)hilensis oysters for 48 h after contact (23). Sawabe et al. also revealed a tropism of a GFP-tagged virulent strain of \( V.\) \( h\)arveyi for gills in the abalone \( Haliotis\) discus \( h\)annah (24). Our results add another degree of clarification and functional complexity regarding the observations made by Travers et al., who visualized an accumulation of \( V.\) \( h\)arveyi on the surface of the gills in infected animals (5). This region may constitute the portal of entry of \( V.\) \( h\)arveyi in the European abalone. The proximity of this targeted region to the heart and the anterior aorta may allow the pathogen to quickly invade the circulatory system of the European abalone (25, 26). This hypothesis of an entry route of \( V.\) \( h\)arveyi via the epithelial region between the gills and the hypobranchial gland could be confirmed using some additional advanced electron microscopy techniques, which may also specify the strategy of \( V.\) \( h\)arveyi entry into gill tissue via an intra- or extracellular route.

In the current study, we have used and tested a TaqMan probe developed by Schikorski et al. (2013) for qPCR assays of \( V.\) \( h\)arveyi (16). Measurements obtained by TaqMan qPCRs following a scheme of serial dilution of a pure culture of \( V.\) \( h\)arveyi in samples of hemolymph or tissues showed an excellent linear correlation, and TaqMan qPCRs have been proven to be powerful and sensitive techniques for \( V.\) \( h\)arveyi detection and quantification in European abalones. TaqMan qPCRs have previously been useful for the detection of pathogenic strains of \( V.\) \( v\)ulgurificus, \( V.\) \( p\)araaeromonas, and \( V.\) \( a\)estuarianus in marine mollusks (27–31). Nevertheless, in our measurements we observed that the counts obtained by qPCR were higher than the counts obtained by the plating method. This observation may be due to the quantification of dead or uncultured bacteria by qPCR.

Quantification of the adhered and penetrated \( V.\) \( h\)arveyi organisms in abalone organs (i.e., gills-hypobranchial gland, digestive gland, and foot muscle) and in hemolymph has elucidated the infection dynamics. After 1 h of contact, \( V.\) \( h\)arveyi was already detected by plating and by qPCR on the surface of all the abalone tissue analyzed, hence inferring that \( V.\) \( h\)arveyi is able to adhere to abalones during the first hour of contact. The concentration of \( V.\) \( h\)arveyi in the hemolymph increased progressively between 3 h and 24 h after contact. This observation suggests that the concentration of \( V.\) \( h\)arveyi in hemolymph may be a proxy for the development of infection in the European abalones and that the stage of multiplication and invasion may occur during the first hours after contact. The rapidity of \( Vibrio\) invasion was also observed in the oyster \( Crassostrea\) gigas, in which virulent strains of \( V.\) \( s\)plendida and \( V.\) \( a\)estuarianus were detected by qPCR in hemolymph after 2 h of contact, and the concentrations of both pathogens were maximal after 6 h (32). On observation of all the organs analyzed here, the density of \( V.\) \( h\)arveyi increased significantly with contact duration in the gills, in which the density was maximal after 6 h of contact. This increase in pathogen density may be due to the local multiplication of \( V.\) \( h\)arveyi on the surface of the gills and the hypobranchial gland, which supports the hypothesis of a putative portal of entry of \( V.\) \( h\)arveyi in this region.

In conclusion, \( V.\) \( h\)arveyi seems to have the capacity to adhere to and penetrate the European abalone during the first hours of contact. We hypothesize that the portal of entry of \( V.\) \( h\)arveyi is the epithelial region situated between the gills and the hypobranchial gland. Further observations need to be conducted to verify the putative portal of entry of \( V.\) \( h\)arveyi in the European abalone and localize it precisely. The invasion of \( V.\) \( h\)arveyi in abalone organs is fast and progressive during the first hours of contact and may be characterized by quantification in the hemolymph. In this study, we have also successfully tested a TaqMan qPCR assay for \( V.\) \( h\)arveyi quantification in European abalone tissue which may be useful for pathogen detection in the natural environment and in farmed stocks. This article demonstrates the importance of pathogen concentration measures during the first hours of contact with the host, improving our knowledge and understanding of the infection dynamics of \( Vibrio\) pathogens in marine mollusks.

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REFERENCES


Infection Dynamics of *V. harveyi* in *H. tuberculata*


