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Laser Capture Microdissection of *Vibrio splendidus* from *Haliotis rufescens* during a post-spawning mortality episode, an alternative method for the study of diseases in aquaculture

Microdisección por captura láser de *Vibrio splendidus* de *Haliotis rufescens* durante un episodio de mortalidad post-desove, un método alternativo para el estudio de enfermedades en acuicultura

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ABSTRACT | Histopathological examination allows direct observation of tissue structure, the presence of pathogens, and their association with cellular and tissue alterations; however, it is almost impossible to isolate and identify these microorganisms from histological preparations. The advent of laser capture microdissection (LCM) technologies in conjunction with molecular tools is currently being used for genomic, transcriptomic, and proteomic studies in human and veterinary pathology and parasitology. A combination of these techniques may be used to identify a particular pathogen. However, this innovative approach has rarely been used to study pathogens in aquatic organisms. Clinical, histological, LCM, PCR, Sanger sequencing, and conventional bacteriological analysis of red abalones, *Haliotis rufescens*, from a broodstock suffering from a post-spawning mortality event were carried out. In all cases, the clinical analysis showed swelling of muscle tissues, visceral mass and excessive mucus production. The histopathological analyses showed curved rod-shaped bacteria affecting the muscle tissues around the mouth, head, foot muscle, and endothelium of haemolymphatic vessels. Analysis by LCM, PCR, and Sanger sequencing allowed the bacteria in the affected tissues to be identified as *Vibrio splendidus*. On the other hand, conventional bacteriological and molecular analysis of a subsample of the head and muscle of one of these abalones allowed the following species to be isolated and identified: *V. splendidus*, *V. chagasii*, *V. mediterranei*, *Vibrio* sp., and *Pseudoalteromonas* sp. LCM technology allowed the detection of the bacterial species proliferating in the tissues and allowed their differentiation from those isolated from whole fresh tissues. This combination of modern techniques helps to define which bacteria should be selected to carry out Koch's postulates and to establish their role in the development of a disease and/or mortality event. The results suggest a detailed study on the role of *V. splendidus* in post-spawning mortality events in *H. rufescens*.

Palabras clave
Histopatología
Abulón rojo
Vibrio splendidus
Microdisección láser

RESUMEN | El examen histopatológico permite la observación directa de la estructura del tejido, la presencia de patógenos y su asociación con alteraciones celulares y tisulares; sin embargo, es casi imposible aislar e identificar estos microorganismos de preparaciones histológicas. El advenimiento de las tecnologías de microdisección por captura láser (LCM) en conjunto con herramientas moleculares se está utilizando actualmente para estudios genómicos, transcriptómicos y proteómicos en patología y parasitología humana y veterinaria. Una combinación de estas técnicas se puede utilizar para identificar un agente patógeno particular. Sin embargo, este innovador enfoque rara vez se ha utilizado para el estudio de patógenos en organismos acuáticos. Se llevó a cabo un análisis clínico, histológico, LCM, PCR, secuenciación Sanger y bacteriología convencional de abulones rojos, *Haliotis rufescens*, de un stock de reproductores que padecían un evento de mortalidad después del desove. En todos los casos el análisis clínico mostró hinchazón de los tejidos musculares, de la masa visceral y producción excesiva de moco. El análisis histopatológico mostró bacterias, con forma de bacilo curvado, que afectaban los tejidos musculares alrededor de la boca, la cabeza, el músculo del pie y el endotelio de los vasos hemolinfáticos. El análisis por LCM, PCR y secuenciación Sanger permitió identificar a las bacterias en los tejidos afectados como *Vibrio splendidus*. Por otra parte, el análisis bacteriológico y molecular convencional de una submuestra de la cabeza y músculo de uno de estos abulones permitió aislar e identificar a las siguientes especies: *V. splendidus*, *V. chagasii*, *V. mediterranei*, *Vibrio* sp. y *Pseudoalteromonas* sp. La tecnología LCM permitió la detección de las especies bacterianas proliferando en los tejidos y permitió su diferenciación de aquellas aisladas de los tejidos frescos completos. Esta combinación de técnicas modernas ayuda a definir qué bacterias deben seleccionarse para realizar los postulados de Koch y establecer su papel en el desarrollo de una enfermedad y/o evento de mortalidad. Los resultados obtenidos sugieren un estudio detallado sobre el papel de *V. splendidus* en eventos de mortalidades post desove en *H. rufescens*.

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INTRODUCTION

Laser capture microdissection (LCM) techniques have been traditionally utilized in pathology, particularly in oncology studies to isolate pure cell populations and even individual cells from heterogeneous tissues. When combined with genomics, transcriptomics, and proteomics, LCM has enabled in-depth studies on cancer development that would not have been possible in the past (Okuducu *et al*. 2005, Liu 2010). Currently, a combination of these techniques has allowed unexpected advances in studies of human and veterinarian pathology (Bogaert *et al.* 2010, Liu 2010, Lu *et al.* 2012, Morton *et al.* 2014, Seclaman *et al.* 2019, Chankeaw *et al.* 2021, Marcu *et al.* 2021, Cruz-Flores *et al.* 2022, Guo *et al.* 2023, Göcz *et al.* 2025). In recent years, LCM has been used to identify non-cultivable bacteria from dissected colonies from histological sections of patients with a histopathological diagnosis of human intestinal spirochetosis (Klitgaard *et al.* 2005). This technique was also used for the isolation of dinoflagellate (*Hematodinium* spp.) parasites from formalin-fixed, paraffin-embedded tissue sections of crustaceans (*Cancer pagurus* and *Portunus trituberculatus*) (Small *et al.,* 2008), although the use of this technique in aquaculture species is minimal. Friedman and Crosson (2012) used LCM to confirm that morphologically different inclusion bodies corresponded to *Candidatus Xenohaliotis californiensis* (*C*Xc), a parasite of abalone species (*Haliotis* spp.). In addition, Cruz-Flores *et al*. (2015) used LCM to separate and confirm that *C*Xc membrane-bound vacuoles could be isolated by filtration. In Atlantic salmon, LCM was used to study myocardial lesions associated with the piscine myocarditis virus (Wiik-Nielsen *et al.* 2012).

It is known that some mortality events in bivalves and gastropods may be related to energy deficiency during the reproduction period and physiological stress associated with reproduction and/or pathogens (Perdue *et al.* 1981, Cheney *et al.* 2000, Friedman *et al.* 2005, Soletchnik *et al.* 2005, Samain *et al.* 2007, Li *et al.* 2009, Cáceres-Martínez *et al.* 2017). The production of gametes and their release may result in an energy loss that favors the susceptibility of organisms such as oysters and abalone to infectious diseases (Travers et al., 2009, Li et al. 2010). Some species of *Vibrio* are considered among the most important opportunistic bacterial pathogens causing diseases in the aquaculture of marine organisms (Ina-Salwany *et al.* 2019). Particularly, in the case of abalone species, *V. harveyi* has been related to massive mortality episodes of *H. tuberculata* along the French coast since 1997 during the reproductive period (Nicolas *et al.* 2002, Cardinaud *et al.* 2014). *Vibrio splendidus* has been involved in the summer mortality of the Pacific oyster *Magallana gigas* (Garnier *et al.* 2007). This study aimed to determine whether bacteria could have been associated with a postspawning mortality event in *H. rufescens* that occurred at an aquaculture facility in Baja California, Mexico, by using LCM and molecular analysis utilizing the 16S rRNA gene.

MATERIALS AND METHODS

Early in March of 2016, at an aquaculture facility near Ensenada City, Baja California, Mexico, 64 red abalone *Haliotis rufescens,* after spawning, started to show particular clinical signs of disease, such as reduced appetite, lethargy, disorientation, muscle spasms consisting of abnormal shell movements, stretching of the foot muscle and head, exiting the tank, swelling of all muscle tissues and the visceral mass, excessive mucus production and, in some cases, death (Figure 1). The water temperature was ≈15°C, and there were no additional parameters for water quality data. The abalones were part of a larger breeding program at the facility and were fed with fresh seaweed (*Macrocystis pyrifera*) and induced to spawn and maintained under regular operational conditions of the aquaculture facility (Searcy-Bernal *et al*. 2010).

A sub-sample corresponding to four of these abalones (size 11.23 ± 0.64 cm of shell length) was transported live in an ice box to the laboratory for histopathology, LCM, bacteriology, and molecular analysis.

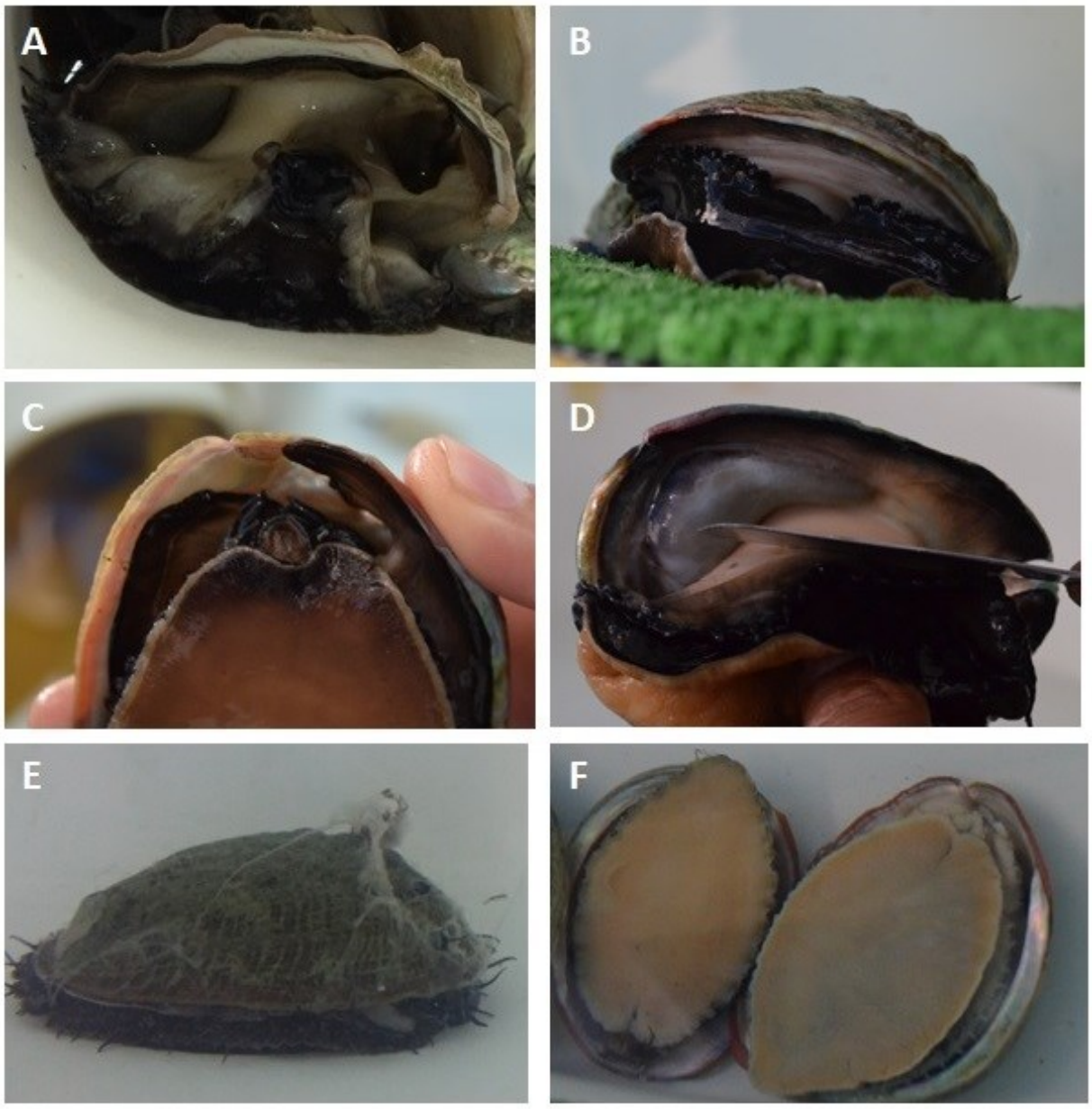

Figure 1. (A) Red abalone *Haliotis rufescens*, lifting the shell and stretching the head. (B) Abalone crawling to exit the culture pond. (C) Foot muscle, head and mantle swelling. (D). Visceral mass swelling. (E). Excessive production of mucus. (F). Dead abalones.

Bacteriological analysis and molecular identification of bacterial strains

The head of one of the diseased abalone was removed aseptically and washed with a solution of sterile seawater and 1% Tween 80. The head was scraped on Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) agar and marine agar for bacterial isolation. The different colonies were isolated using the streaking method in 2% Tryptic Soy (TSA) NaCl agar. The isolates were incubated for 24 h at 23.6 ± 2 °C. Isolation was continued until a single type of colony was obtained based on a macroscopic review that included the colonial morphology, coloration, type of growth, and consistency of the colony (Holt *et al.* 1994). Phenotypic characterization was performed with conventional tests considering colony morphology and Gram staining. For biochemical characterization, cytochrome oxidase and catalase tests were used. Once the bacteria were isolated and identified, they were preserved at -20 °C following the methodology proposed by Sambrook and Russell (2001).

Histopathology and capture laser microdissection (LCM) analysis

Fractions of the cephalic tissue, foot muscle, and visceral mass of the three abalones showing signs of disease were processed by histology. The tissue samples were approximately 5 mm thick, placed in histological cassettes, and dehydrated following the protocol of Howard and Smith (2004). Subsequently, they were embedded in paraffin and sectioned at 5 µm. These sections were mounted on histological slides and stained with hematoxylin-eosin (Shaw and Battle, 1957). The histological slides were reviewed with a light microscope (Axioplan 2, Carl Zeiss) at different magnifications to look for bacteria and other tissue alterations. The paraffin block containing the tissue where bacteria were observed on histological slides was selected for LCM analysis. A new sample was sectioned from the histological block and mounted on a PEN membrane glass slide (Life Technologies). The slide was stained using the Arcturus® Paradise® PLUS Reagent System. Bacteria were dissected using an ArcturusXT Laser Capture Microdissection system.

For molecular characterization of bacteria obtained from the fresh tissues and LCM, partial sequencing of the 16S rRNA gene was performed. Following the manufacturer's recommendations, DNA extraction of bacteria dissected from histological slides was performed using an Arcturus® Pico Pure® DNA extraction kit. For fresh tissues, the isolates were placed in sterile 1.6 mL microcentrifuge tubes. DNA extraction was performed using the DNeasy Blood and Tissue Kit (Qiagen) kit following the manufacturer's instructions. In both cases, PCR analysis was carried out using the following primers: 16S forward (5´-CCGTCGACAGAGTTGATCCTGGCTCAG-3´) and 16S reverse (5´-CGGGATCCACCTTGTTACGACTTCACCC-3'), which amplify a 1500 bp fragment and are universal for most eubacteria (Weisburg *et al.* 1991)

The final volume of the PCR was 50 µL using 200 µM dNTP, 20 nM of each primer, 1.5 mM $MgCl_2$, and 1.25 U Taq DNA polymerase (Weisburg *et al.* 1991). PCR was performed in an Apollo thermocycler. The amplification conditions were as follows: initial denaturation at 95 °C for 5 min; 25 cycles at 95 °C for 1 min., 55 °C for 1 min, and 72 °C for 2 min; and a final extension at 72 °C for 10 min. In all assays, a negative control consisting of sterile water was included. All samples were run in duplicate. PCR products were visualized by gel electrophoresis on a 1% gel. A 100-V charge was applied for 1 h and stained with a Red® Gel solution. A molecular weight marker of 0.1 to 1 kb (Bioline) at a concentration of 1 µg/µL was used as a reference. The amplified PCR products were sequenced directly in both directions at Etonbio Biosciences, Inc., Laboratory in San Diego, California (USA). The sequences obtained were manually edited using Mega 7.0 software (Tamura 2007). The sequences were compared using BLASTn, and a 98.65% similarity was used to determine species (Kim and Chun 2014).

RESULTS

Histopathology

Two abalones were sexually undifferentiated with empty follicles, and two were females with some oocytes attached to the follicles. In all 4 abalones, abundant brown cells were observed in the muscle tissue surrounding the mouth, as well as in the foot muscle (Fig. 2 A, B). Clumps of bacilli-form bacteria close to brown granules were observed. Some bacteria in these clumps seemed to be dispersing from the clumps and granules (Fig. 2 CD). In other cases, a large number of bacteria appeared to be leaving the focal clumps into the muscle tissue (Figure 2 E). These bacteria were also observed adhering to the endothelium of the haemolymphatic vessels (Figure 2F). In the muscle tissue, areas with a large quantity of bacteria and tissue destruction were observed (Fig. 3 A, B).

Laser capture microdissection (LCM)

The abalone selected for bacteriological and LCM analysis was undifferentiated. Figure 3 A-B shows bacilli-shaped bacteria observed in the foot muscle tissue via conventional hematoxylin-eosin staining. Figure 4 A-B shows the twin area of the tissue showing these bacillary bacteria in unstained tissue where LCM was performed. LCM areas where the dissection was performed showed empty spaces and fractions of the dissected tissue with the bacteria used for DNA identification (Fig. 4 C, D). Bacteria dissected directly from the tissue using LCM could correspond to a strain of *V. splendidus* with 99.86% identity (similar to GenBank accession number KM652199.1).

Bacteriological analysis

All bacteria isolated from the cephalic region using conventional bacteriological identification were Gram-negative, bacilliform, and catalase and oxidase-positive; four of the isolates corresponded to the genus *Vibrio,* and one belonged to the related order Alteromonadales based on BLASTn analysis (Altschul et al. 1990) (Table 1).

Table 1. Sequence relatedness of the bacterial strains found in diseased abalone to bacterial sequences deposited in GenBank by BLASTn analysis.

GenBank accession number	Bacterial species isolated in diseased abalone	Coverage (%)	Identity (%)
KM652199.1	*Vibrio chagasii*	99	89
AJ874367.1	*Vibrio splendidus*	99	99
CP018308.1	*Vibrio mediterranei*	99	98
KP770087.1	*Vibrio* sp.	99	99
MT645493.1	*Pseudoalteromonas* sp.	100	90

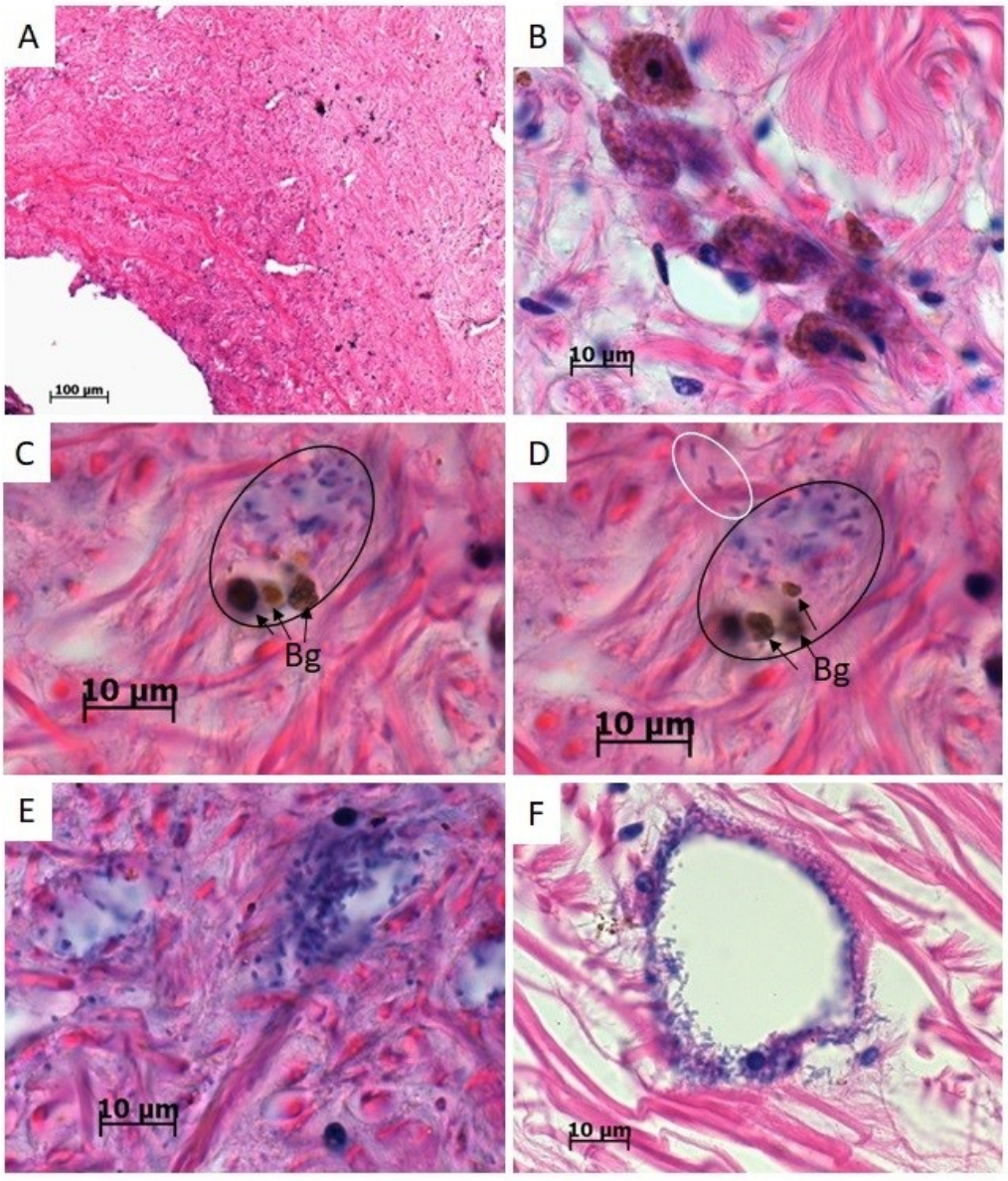

Figure 2. Images of the muscular tissue of red abalone *Haliotis rufescens* affected by *Vibrio splendidus*. A. Wide view of the muscular tissue of the mouth with several dark dots that correspond to brown cells. B. Close-up of the brown cells among muscular tissue. C. A clump of bacilli bacteria (black circle) is shown close to brown granules (Bg). D. Another plane of panel C where some bacteria (circle) seem to have been dispersing from the clump of bacteria and brown granules (black circle). E. Numerous bacteria dispersed into the muscular tissue from focal clumps of bacteria. F. Numerous bacteria around the endothelium of a haemolymphatic vessel.

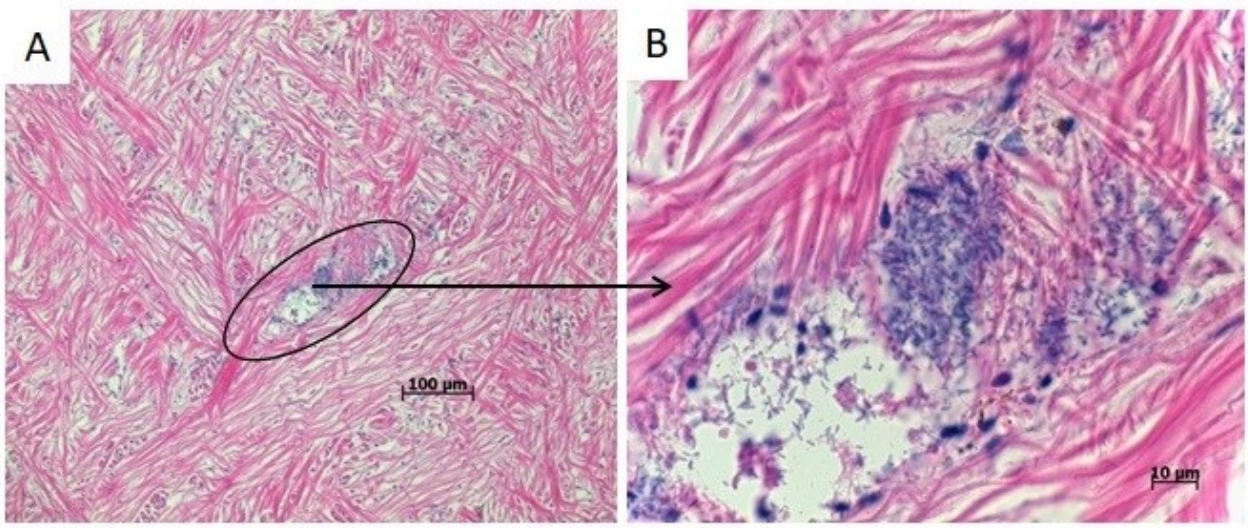

Figure 3. A. General view of the foot muscle of a red abalone *Haliotis rufescens* where bacterial growth was observed among muscle fibers (encircled). B. Close-up of infected muscle where numerous curved bacilli of *Vibrio splendidus* were growing.

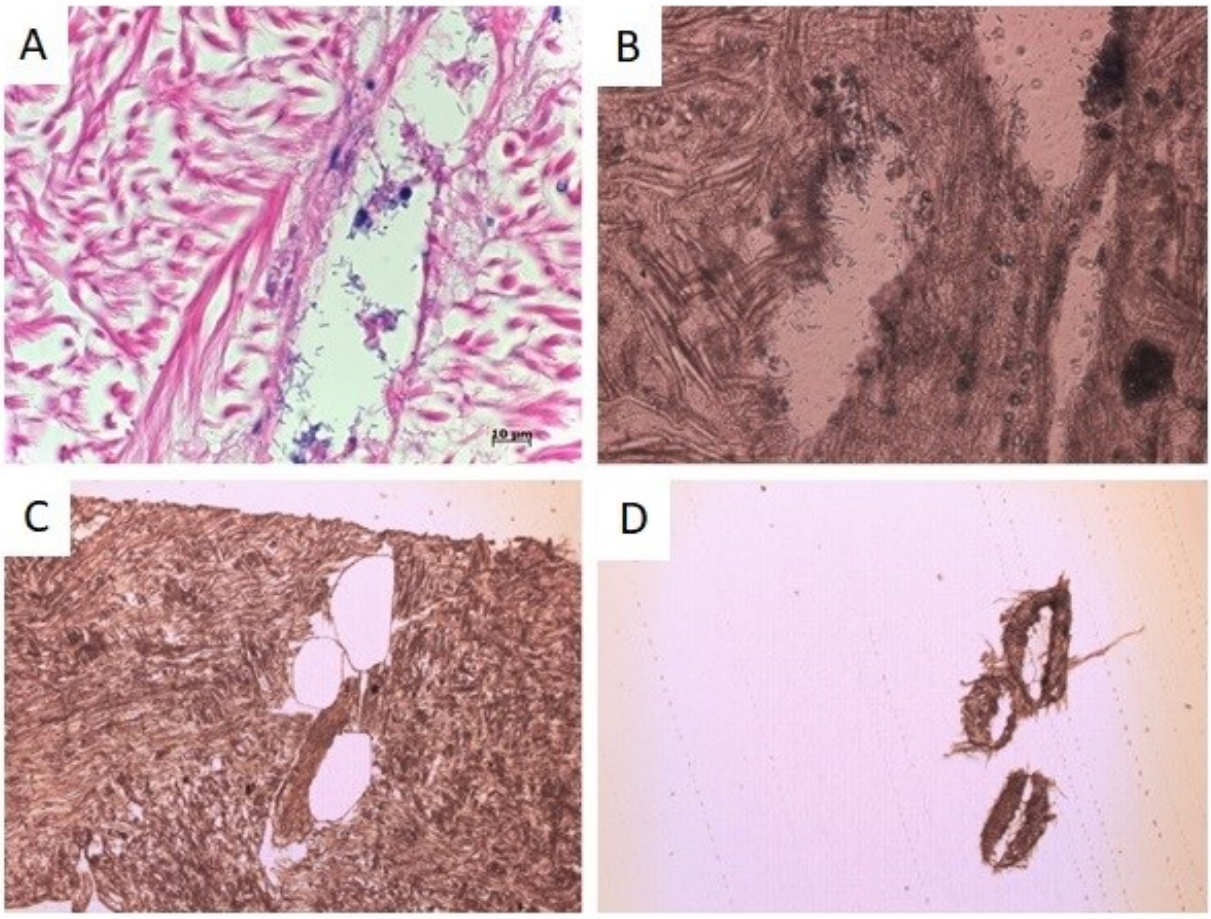

Figure 4. Images of the muscular tissue of red abalone *Haliotis rufescens* affected by *Vibrio splendidus*. A. Muscle tissue stained with hematoxylin-eosin where curved bacilli bacteria were observed. B. Unstained twin areas of the tissue where the same bacteria were observed. C. Empty areas in the foot muscle where tissue was cut using laser microdissection. D. Fractions of microdissected tissues used for DNA extraction of bacteria and posterior molecular identification.

DISCUSSION

Laser capture mirodissection (LCM) analysis, in combination with molecular methodologies, allowed determine the presence of a putative strain of *V. splendidus* in abalone tissues and to associate this bacterium with defined cellular and tissue alterations. This study showed successful DNA extraction from tissues obtained by LCM and the subsequent amplification of the complete 16S rRNA gene. A parallel conventional bacteriological study revealed the presence of *V. splendidus*, *V. chagasii*, *V. mediterranei*, *Vibrio* sp., and *Pseudoalteromonas*. It is important to note that while sequencing the near-complete *16S rRNA* gene provides reliable bacterial identification, complementary analysis using additional genes, such as the *23S rRNA* and *rpoB*, is recommended for enhanced accuracy. As mentioned earlier, it is known that after spawning, the susceptibility of bivalve and gastropod mollusks to bacterial infections may increase (Perdue *et al.* 1981, Cheney *et al.* 2000, Friedman *et al.* 2005, Soletchnik *et al.* 2005, Delaporte *et al.* 2007, Samain *et al.* 2007, Li *et al.* 2009, Li *et al.* 2010, Cáceres-Martínez *et al.* 2017). In this study, the follicles of two abalones were empty, and the others showed few residual gametes attached to the follicles, which corroborated the post-spawn condition. It is known that *Vibrio* spp. forms part of the bacterial community found in coastal waters where aquaculture operations of bivalve mollusks and other marine organisms occur (Allam *et al.* 2002). When environmental conditions are favorable, species such as *V. tasmaniensis, V. splendidus*, and *V. neptunius* may cause diseases in aquatic organisms (Thompson *et al.* 2003, Guisande 2004). In the European abalone, *V. harveyi* has been related to disease and mortalities in France associated with temperature changes and the reproductive stage (Nicolas *et al.* 2002; Travers et al. 2009; Cardinaud *et al.* 2014). Disease outbreaks of cultured blacklip abalone *Haliotis rubra* and greenlip abalone *Haliotis laevigata*, including its hybrids, have been related to *V. harveyi*, *V. splendidus*, and a *Flavobacterium*-like bacterium (Handlinger *et al.* 2005).

Among the bacterial species detected in the present study, *V. chagasii* was described by Thompson *et al*. (2003) and was isolated from the gut of turbot larval *Scophthalmus maximus*. *Vibrio mediterranei* was described by Pujalte and Garay (1986) and was isolated from marine water, sediments, and plankton in the coastal area of Valencia, Spain. This species has been considered synonymous with *V. shiloi* (Thompson *et al.* 2001). In addition, *V. chagasii* has been considered pathogenic to *Patinopecten yessoensis* (Wei-Ming *et al.* 2012) and was involved as part of the changes in the microbiota associated with juvenile abalone *Haliotis discus hannai* during a disease outbreak in southern China (Shi *et al.,* 2017); in the present study, it was found in fresh tissues of the head, but it was not found by sequencing the DNA obtained by LCM, suggesting that it could be a secondary factor in this event. The presence of *V. splendidus* in the fresh tissue of the head and from LCM suggests that this species could be involved in the disease of studied abalone. Interestingly, histopathological analysis revealed part of the process of dispersion of *V. splendidus* in the muscle tissues and their degradation. Similar observations were noted by Garnier *et al*. (2007) in the Pacific oyster *M. gigas* suffering summer mortalities in which *V. splendidus* was involved. The presence of these bacteria in the endothelium of haemolymphatic vessels suggests their possible spread to all tissues, resulting in bacteremia. The histopathological lesions could have resulted in the observed external signs of disease, such as muscle spasms resulting from abnormal shell movements, stretching of the foot and head, and swelling of all muscle tissues. However, it is known that *Vibrio* spp. are common abalone gut microbiome members (Sawabe 2006) and that invasion of dying or dead abalone by *Vibrio* spp. may be possible regardless of the cause of death.

To determine with certainty that *V. splendidus* was the etiological agent of these mortalities, a major sampling of diseased abalones would be desirable. In addition, it is necessary to carry out Koch's postulates in post-spawned and unspawned organisms in similar environmental conditions, particularly at similar temperatures. The identification of this bacterial species in fresh tissues and in tissues isolated by LCM and considering its role as an important pathogen for abalone and other marine organisms places this bacterium as a strong candidate for selection for Koch's postulates over the other identified bacterial species. *In situ* hybridization is more commonly used to detect specific pathogens in histological tissues; however, LCM is more powerful by allowing the sequencing of relevant genes of the pathogen, which provides more information than ISH alone. LCM technology aids in the selection of particular species suspected to be the cause of a certain disease and allows its relationship with specific tissue alterations.

Credit author statement (CrediT)

Rebeca Vásquez-Yeomans: Investigation, Methodology, and Writing- original draft: *Jorge Cáceres-Martínez:* Investigation, Methodology, and Writing. *Roberto Cruz-Flores:* Investigation, Methodology, Writing - review & editing.

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