Vibrio mediterranei, a Potential Emerging Pathogen of Marine Fauna: Investigation of pathogenicity using a bacterial challenge in *Pinna nobilis* and development of a species-specific PCR.

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Running Page Head: Vibrio mediterranei challenge in Pinna nobilis

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Abstract

Aims: Extreme mortality events affecting *Pinna nobilis,* some associated to *Vibrio mediterranei,* have depleted many populations of this bivalve. The objective of this study was to demonstrate pathogenicity of *V. mediterranei* in the host *P. nobilis* by performing a bacterial challenge in *P. nobilis* to understand if *V. mediterranei* has specific virulence in this host. To assist this objective, a secondary objective was to develop a species-specific DNA diagnostic test.

Methods and Results: *P. nobilis* collected from local bays were used in a challenge experiment with *V. mediterranei* (strain IRTA18-108). Virulence in the host background of *P. nobilis* was demonstrated at doses of 10^3 CFUs / animal. An alignment of published *Vibrio spp.* atpA sequences was used to design *V. mediterranei*-specific primers. Further, data mining of published literature and *V. mediterranei* genomes identified multiple virulence-related genes (*vir* genes) from which specific primers were designed for PCR detection of selected genes.

Conclusion: *V. mediterranei* strain IRTA18-108 is pathogenic in the host *P. nobilis*. The virulence genes *sod, rtx*, and *mshA* were identified in this strain. Temperatures of 24ºC or higher appear to trigger onset of virulence. Sensitivity and specificity of the Vm atpA PCR is useful for diagnosis of Vibriosis in shellfish.

Significance and Impact of the Study: The presence of previously described virulence genes have been confirmed in this strain. The specific Vm atpA PCR assay will aid management of future epizootics of this emerging pathogen of aquatic fauna, and improve surveillance capabilities for mortality events where Vibrios are suspect.

Keywords: shellfish, *Vibrio mediterranei, shiloi, shilonii Pinna nobilis* Mediterranean, PCR diagnostic

Introduction

Emerging diseases are more of a threat when not anticipated, and even more so in this era of climate change where even conservative estimates show global temperatures will rise significantly in the coming years (Collins et al., 2013). Rising water temperatures have been shown to play a determinative role in the life histories of various aquatic animal pathogens (virus, bacteria and parasites) where specific threshold temperatures need to be surpassed for activation of their metabolism, trigger expression of virulence mechanisms or promote their reproductive life cycle (El Matbouli et al., 1999; Moore et al., 2002; Carrasco et al., 2017). With changing environmental conditions, the triad of host-pathogen-environment shifts in such a way as it can favor some pathogens so that they move from being merely opportunistic pathogens to “true” pathogens.

Among the genus *Vibrio* there are many well-described pathogens of aquatic fauna: fish, shrimp, coral, and bivalves (among others are *Vibrio anguillarum, Vibrio harveyi, Vibrio coralliilyticus*, and *Vibrio aestuarianus*, respectively for each host) (Abraham et al., 1999; Ben-Haim et al., 2002; Pang et al., 2007; Romalde et al., 2014; Goudenège et al., 2015). In some reports, *Vibrio mediterranei* has been found associated to aquatic animals such as corals and scallops, and was considered as a potential pathogen (Rubio-Portillo et al., 2014; Serrano et al., 2018). The ability for *V. mediterranei* to act as a pathogen may be related to quorum-sensing mechanisms as has been shown with experiments in brine shrimp and manila clams (Torres et al., 2018). Virulence mechanisms related to adhesion, superoxide dismutase production and toxin production (Reshef et al., 2008) have also been described from *V. mediterranei*. Additional virulence genes encoding pili and zona occludins toxin (Zot) have also

been found in whole genome screening (Reshef et al., 2008) from *V. shiloi* (a synonym of *V. mediterranei* – see below). There are a few mentions of *V. mediterranei* associated to morbidity and /or mortality in *Pinna nobilis,* the giant fan mussel (Rodríguez et al., 2017; Prado et al., 2020), but the specific etiology of *V. mediterranei* in this host has not been investigated.

Other pathogens have been described as having a tremendous impact on populations of *P. nobilis*. After an extensive epizootic of *Haplosporidium pinnae* that devastated *P. nobilis* populations along much of the coast of the Spanish Mediterranean coast (Catanese et al. 2018), as well as other coastal areas of the Mediterranean (Carella et al., 2019; Katsanevakis et al., 2019), a project was initiated in 2017 for species protection to aid recovery of a significant population in Alfacs Bay (northwestern Mediterranean Sea) of *P. nobilis* (Prado et al., 2020). For this, a permit was obtained to collect 106 individuals into captivity for protection from disease, although after the shellfish were collected from Alfacs Bay and had acclimated to aquaria conditions some individuals showed signs of morbidity and several ultimately died. The mortality episode progressed among the captive animals during part of 2018 into early 2019, during which cumulative death showed a strong association with temperature, with losses starting at water temperatures of ca. 19 °C and peaking during the summer months when temperatures reached 25- 26 °C, in agreement with temperature preferences for expression of virulence phenotypes in *V. mediterranei* (Vattakaven et al., 2006). From 3 moribund individuals, tissue samples were collected for bacteriological assessment, and among 19 Vibrio colonies collected from TCBS agar plates, 16 were identified as *V. mediterranei* using 16 S ribosomal DNA and *atpA* sequencing followed by phylogenetic analysis. Regionally shellfish diseases have

been dominated by mass mortalities of oysters, and oyster herpes virus (OsHV) has been an endemic problem affecting commercial oyster populations in the Alfacs Bay area since 2008 (Carrasco et al., 2017). Other pathogens may also be implicated since many episodes of unexplained mortalities in oysters still occur (unpublished data). Therefore, diagnostic PCR for OsHV, *Vibrio aestuarianus, V. splendidus*, and *H. pinnae* were already in use for molecular diagnostics of shellfish diseases in our lab, but when performed using DNA extracts of tissue from affected *Pinna* individuals no positive results were obtained, therefore with respect to *P. nobilis*, attention has become focused on the Vibrio isolates.

The estimated value for fisheries production for 2016 was USD 232 billion with significant losses being seen in recent years due to emergent diseases (FAO, 2018). Specific mention has been made by the FAO advocating the need for improved diagnostics for emerging diseases to improve rapid reporting of disease outbreaks for better management of response to epizootics, and to improve spatial planning of new aquaculture operations. Assays using PCR have clear advantages over DNA sequencing to facilitate rapid decision making in the face of major epizootics.

Specific identification is always of concern when attributing etiology to a species of bacteria. The coral pathogen *V. shiloi* (Kushmaro et al., 2001) has been identified as a later synonym of *V. mediterranei* (Pujalte et al., 1986; Thompson et al., 2001; Tarazona et al., 2014). Contributing to confusion over correct identification is the accession number for the 16S sequence ascribed to *V. shiloi* [AF007115] (Kushmaro et al., 2001); when entered into a search of the GenBank database, this number brings up the sequence for *Vibrio shilo**nii***;

different binomial nomenclature but in fact the same species. The scientific literature has progressed in recent years under the guise of all 3 names.

Bacterial systematics has come to rely heavily on phylogenetic analyses of ribosomal genes, among other “house-keeping“ genes. The 16 S ribosomal RNA gene has a high degree of conservation within some genera of bacteria, notably Vibrio. For this reason multi-locus sequence typing (MLST) is often used for correct taxonomic assignment (Sawabe et al., 2013; Tarazona et al., 2014). Depending on the taxa in question, specific small loci of sequence motifs have also proven to be useful for differentiation among closely related bacterial species (Lal et al., 2013), and leading some researchers to utilize specific house-keeping genes such as *atpA* for phylogenetic assessment and taxonomic assignment (Thompson et al., 2001; Thompson et al., 2007; Lal et al., 2013). In a previous study of *V. harveyi*, the *atpA* gene was described and characterized (Lal et al., 2013), and it was demonstrated that *atpA* can serve as a reliable marker for phylogenetic identification of multiple species, though not all Vibrio spp.

Given the risks of the convergence of climate change and emergent pathogens of aquatic fauna, the work herein considers the species *V. mediterranei* as an emergent threat, and therefore in need of specific, rapid diagnostics for surveillance and epidemiologic study. By comparing the *atpA* sequence from *V. mediterranei* with the *atpA* sequences described in GenBank for other Vibrio species, a variable region was identified that enables specific detection of *V. mediterranei*. Moreover, while association of *V. mediterranei* to mortalities of *P. nobilis* have been reported previously (Rodríguez et al., 2017; Prado et al., 2020) the specific pathogenicity of *V. mediterranei* for this host has not been demonstrated. To this end, permits were obtained to collect a limited

number of this strictly protected species to be recovered from the wild to identify the specific risks of *V. mediterranei* using a challenge experiment. The present study´s aim is to demonstrate pathogenicity of *V. mediterranei* (strain IRTA18-108), previously recovered from moribund *P. nobilis* held in captivity, using the original host from which it was isolated, thus fulfilling most of Koch´s postulates. Results from this challenge experiment will be of use in estimating the risks of the presence of this bacteria to populations of *P. nobilis*. To enable this work a specific diagnostic test was needed. Therefore, a secondary objective was demonstration of the utility of a PCR assay for detection of the *V. mediterranei atpA* sequence for identification of *V. mediterranei*. In the present work we focus this study on mortalities occurring in the endangered giant fan mussel, *P. nobilis*, where *V. mediterranei* was previously associated to moribund and dead animals.

Materials and Methods

Sample collection

A total of 52 *P. nobilis* individuals were collected from sites within Alfacs Bay (n= 52) [40º 35´ 40.59´´N; 0º 39´ 37.36´´E] to be used in a bacterial challenge experiment (see below). From this total, 10 animals were analyzed by Vm atpA PCR to determine the baseline level of contamination by *V. mediterranei,* and the remaining 42 animals were used for the bacterial challenge (see below). The *P. nobilis* animals were all juveniles appearing to be of the same year class, and therefore of quite similar size. Shell length and width was measured to the nearest mm. Weight was not evaluated due to large differences among individuals in water content, therefore this metric was not considered valid for comparison.

Given the rapid growth rate of the species it could be seen the animals collected belonged to the same year class and should be similar in terms of their immune status and development. Additional adductor muscle tissue samples analyzed by PCR were obtained from a mass mortality event that occurred among captive *P. nobilis* being conserved in aquaria in 2017-18 (Prado et al., 2020). The 16 *V. mediterranei* strains used in this study were collected from the same mortality episode in 2017-18 and their isolation is previously described (Prado et al., 2020).

DNA extraction, PCR amplification, sequencing

Extraction of DNA utilized the Qiagen Blood and Tissue Kit (Qiagen, Madrid, Spain), and all samples were evaluated by spectrophotometry ($A_{260/280}$ ratios) to assess purity and concentration. Primers used for amplification of the gene encoding *atpA* of bacteria are shown (Table 1). Each 25 µL polymerase chain reaction contained 800 µM dNTP´s, 2 mM $MgCl_2$ and 0.6 µM of each primer. The thermal cycler program used, with the primers described in Lal et al. (2013), was 40 cycles of 95 ºC for 1 min, 56 ºC for 30 sec, and 72 ºC for 45 sec, preceded by 5 min at 95 ºC, and followed by 10 min at 72 ºC. The ribosomal 16 S sequence was also obtained for comparison of strains (Prado et al., 2020). In all of the above described PCR reactions DNA samples were normalized to 5 ng/µL for genomic DNA purified from isolated bacteria, or in the case of contaminated tissues, to 50 ng/µL.

For sequencing, each PCR product was purified using the Qiagen PCR Purification Kit (Qiagen, Madrid, Spain), then sent to a private company (Sistemas Genomicos, Valencia, Spain) for bi-directional sequencing using the same primers as those in the original amplification. The resulting sequence data

was edited and trimmed using BioEdit (Hall, 1999) to remove terminal primer sequences and cleaned of any aberrant base-calling before aligning the forward and reverse reads to construct a consensus sequence representative of this strain of the species (Accession # MK471357-MK471368). The sequences were subjected to analysis using BLAST to estimate the identity of the sequence obtained, and further phylogenetic analysis was performed for confirmation (Prado et al., 2020).

After sequencing the *atpA* gene from the local strains of *V. mediterranei*, new species-specific primers were designed which amplified a smaller product of 914 bp. Amplification conditions were the same as described above. These sequences were included in a phylogenetic analysis to confirm results obtained by BLAST analysis.

Phylogenetic analysis

From the original 16 *V. mediterranei* isolates we obtained *atpA* sequences that were aligned with the *atpA* gene sequences from 51 additional species using BioEdit (Hall 1999) to search for i) homology with the *V. mediterranei*-specific primers; ii) the presence of a homologous Sca I site at the specified region within the *V. mediterranei* atpA gene sequence; and iii) demonstrate the isolated strains affiliation with the *V. mediterranei* clade using phylogenetic analyses.

The phylogenetic analyses were performed using MEGA X (Kumar et al., 2018), with the evolutionary history being inferred using the Maximum Likelihood method that included 1000 bootstrap replicates to establish confidence limits (Felsenstein, 1985). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura 1980) and are in the units of the number of base

substitutions per site. The rate of variation among sites was modeled with a gamma distribution (+G, parameter = 0.4242). All ambiguous positions were removed for each sequence pair. The final analysis of Vibrio *atpA* involved 67 nucleotide sequences with a total of 859 positions in the dataset.

Optimization of the V. mediterranei atpA PCR assay

To confirm specificity of the Vm atpA PCR assay, genomic DNA samples from previously verified Vibrio species in our laboratory collection were analyzed. The strain collection is maintained for bacterial challenge experiments and as sources of positive control DNA for diagnostics. This collection includes *V. anguillarum*, *V. alginolyticus*, *V. harveyi*, *V. splendidus*, in addition to *V. mediterranei.* All samples were normalized to a concentration of 5 ng·μL^{-1} of genomic DNA. The sensitivity of the assay was established using DNA extractions from specific numbers of colony forming units (CFU´s) obtained from a logarithmically growing culture. The sample that included 10^7 CFUs provided a DNA sample with a concentration of 56 ng·μL^{-1}. This dilution series therefore ended with a sample of 56 fg·μL^{-1} at the equivalent of 10^0 CFUs (this amount of DNA could not be verified by spectrophotometry as it was below the range of detection). Briefly, a bacterial suspension was prepared in sterile PBS using colonies scraped from TSA plates containing 48 hour cultures. The cell suspension was diluted to an OD_{550} of 0.6 using sterile PBS. Following this, 10-fold serial dilutions were prepared in sterile PBS to obtain specific numbers of cells from 10^7 – 10^0. Cell concentrations were confirmed afterwards by colony counting after 48 hrs incubation of the TSA agar plates at 20 ºC. Aliquots of 1mL from these cell suspensions were centrifuged at 18400 xg for 10 min to pellet cells for DNA extraction. Purification and quality control of purified DNA extracts

were performed as described previously. Aliquots of these same bacterial DNA samples were also tested in the presence of 1 µg of host DNA, which had been demonstrated previously to be free of detectable amounts of DNA from *V. mediterranei*.

Restriction Fragment Polymorphism Analysis

Following amplification and sequencing of the *atpA* gene a unique Sca I site was identified. This Sca I site was exploited to further confirm the identity of amplified products. A mix of 5 µL of amplified *atpA* gene fragment was mixed with an equal volume of water, 2X CutSmart™ Buffer and 1U Sca I restriction enzyme (New England Biolabs, Ref# R3122S). This mixture was incubated at 37 ºC for 3 hours and then the fragments separated on a 1.5 % agarose gel.

Antibiogram of V. mediterranei strains

Sixteen strains of *V. mediterranei,* initially isolated from moribund *P. nobilis* in 2019*,* were screened for differences in antibiotic sensitivities, in addition to the phylogenetic analysis and genetic profiles obtained from the vir gene-specific PCRs (described below), to detect additional possible strain differences among them. All strains were previously stored at -80 ºC in brain heart infusion broth containing 40 % glycerol. Cryo-tubes of stocks were thawed on ice and a 100 µL aliquot spread onto BHA + 3 % NaCl media in petri dishes, then incubated at 20ºC for 24 hours. From these revived stocks, inoculum for each strain was prepared by diluting bacterial suspensions of overnight log phase cultures in PBS 1X to an adjusted OD_{550} of 0.6. From each inoculum 100 µL was spread onto new BHA + 3% NaCl plates and antibiotic discs (norfloxicin-10 mg; oxytetracycline- 30 mg; erythromycin- 30 mg; florfenicol- 30 mg) placed

equidistant within separate quadrants of the plate surface. After incubation for 18 hrs at 20 ºC the diameter of zones of inhibition were measured to the nearest mm.

Challenge Experiment

A permit was granted for the collection of *P. nobilis* from Alfacs Bay for performing the challenge experiment (document identification SF/0510/2019). Within 24 hours of collection of the experimental animals from the bay, 10 animals were sacrificed prior to the challenge to analyze different tissues for contamination with *V. mediterranei*. The *P. nobilis* tissues (branchia/mantle, kidney, adductor muscle, gonad, and digestive gland) were collected aseptically and placed in tubes on ice before freezing at -20 ºC until analysis by PCR. DNA extractions were performed as described above.

The 42 remaining animals were held for two weeks to acclimatize to the ambient conditions of the experimental aquaria. The challenge experiment was performed using rectangular 50 L aquaria of polypropylene plastic that initially were on open flow using sterilized water of ambient temperature. Water was sterilized by ozonation as a standard practice for this facility and this prevented viable bacteria from the bay waters entering into the experimental chamber. At the start of the experiment the water temperature was 20 ºC and increased during the following 23 days since this was the period leading into the spring season. By the end of the experiment water temperature was 24 ºC. The absolute salinity varied slightly during this period with a range between 35.7 ‰ and 37 ‰. Photoperiod was a natural day: night cycle for the month of June at the given GPS coordinates (see above). Although all animals were of the same year class

and similar size, animals were randomly assorted to the various tanks (n= 7/tank) to avoid possible bias to the mortality results due to slight variations in size of the animals. Once introduced to the experimental tanks they were held for an additional two weeks for acclimation to ambient conditions and observe if there were any moribund animals due to transport stress. The experiment was initiated with an injection with 0.1 mL of inoculum of *V. mediterranei* (IRTA18-108), afterwards the system was closed and one third of the water was renewed daily after each feeding. Animals were fed daily as described in Prado et al. (2020).

To begin preparation of inoculum the bacterial strain IRTA18-108 was recovered from glycerol stocks of the purified strains of *V. mediterranei* held in -80 ºC. The cryotube was thawed on ice and 100 µL was inoculated onto an agar plate containing TSA + 3 % NaCl. A single colony from this plate was spread onto a new plate and incubated for 48hrs at 20 ºC, a temperature described as “well within their optimal growth range” (Vattakaven et al., 2006). Colonies from this second plate were suspended in sterile PBS to obtain a suspension with an $O.D._{550}$ of 0.6. This was serially diluted 10-fold and 0.1 mL of each dilution spread onto new plates, incubated 24 hrs at 20 ºC, then colonies counted to obtain a correlation between $CFU´s{\cdot}mL^{-1}$ and the optical density. Following this data, new inoculum was prepared to inject 0.1 mL into the adductor muscle of *P. nobilis*. Given that *P. nobilis* is a protected species with regional populations under increased pressure from an epizootic of *H. pinnae*, the typical experimental design of performing an experiment *a priori* to determine the LD_{50} for establishing the challenge dose was omitted to minimize the number of animals to be sacrificed. There was one tank per dose of inoculum and each tank contained 7 individuals per dose which ranged from 10^7- 10^3 CFU per animal (ie. – injection

of 0.1mL volume of suspensions of 10^8 -10^4 CFU·mL^{-1}) with a control tank within which animals were injected with sterile PBS. Doses injected were confirmed by plate counting. After injection, animals were checked every two hours during 10 hour periods daily to collect dead individuals. From the first 3 animals to die from each challenge dose, samples of tissue (as described above) were collected into Davidson´s fixative for histological analysis, and also for DNA extraction. The samples of excised tissues were placed into 2 mL tubes with approximately 100 mg of 0.5 mm diameter zirconium glass beads to aid in tissue homogenization for DNA extraction. Samples thus prepared were frozen at -20 ºC until analysis. Any remaining animals that died were frozen at -20 ºC for later isolation in culture of *V. mediterranei* on solid media (TSA + 3 % NaCl). For this isolation frozen tissues were thawed on ice. External surfaces were sterilized of extant bacteria by spraying with 70% ethanol. The ethanol was allowed to evaporate for a few moments and then an incision was made in the kidney of the animal. A sterile cotton swab was inserted and passed within the tissue to recover a swab sample that was spread onto TCBS + 3 % NaCl plates for incubation at 20 ºC for 48 hrs. Colonies from this isolation were used for DNA extraction and screening using the new Vm atpA PCR assay.

V. mediterranei PCR testing of Pinna nobilis tissues

Five different tissues (gonad, kidney, adductor muscle, branchia, and digestive gland) were sampled for examining the baseline level of contamination/infection with *V. mediterranei* of those animals collected from the wild for use in a bacterial challenge (see below). Once the challenge experiment was initiated, the first 3 animals to die from each challenge dose were also similarly dissected for tissue collection, DNA extraction and PCR screening for *V.*

mediterranei to confirm presence of the bacteria. The DNA was extracted from these samples as previously described and the amplified DNA was analyzed by agarose gel electrophoresis. Additionally, a retrospective analysis of approximately 50 mg of 30 frozen adductor muscle tissue samples from dead and moribund *P. nobilis* collected during a mortality event of captive animals in 2018-2019 were analyzed by PCR. The *atpA* primers used for specific detection of *V. mediterranei* are those described in this work that generate a product of 914 bp.

Vir gene profile of V. mediterranei strains

Given notable variation in antibiotic sensitivities among some strains, additional genetic screening of strains was performed to validate strain phenotypes and select a likely virulent strain for the challenge trial. Multiple virulence genes have been described for *V. shilonii/V. mediterranei* and whole genomes are available on public databases for downloading the described virulence genes. We analyzed genomic DNA extracted from the 16 strains of *V. mediterranei* for the presence of several virulence related genes including: superoxide dismutase, *sod* accession # ABCH01000140.1; pillin subunit A, *mshA* accession # NZ_BCUE01000002.1; zonular occludens toxin, *zot* accession # FLLQ01000021.1; RTX toxin, *rtx* accession # NZ_BCUE01000015.1; zeta toxin, *zeta* accession # NZ_BCUE01000036.1; outer membrane protein U, *ompU* accession # EDL55710.1; toxin R, *toxR* accession # EDL54045.1. Specifc primers for amplification of fragments of 165- 355 bp for detection of these genes are shown (Table 1). Amplification conditions were the same as those shown above for the *atpA* fragment.

Results

The species of Vibrio identified from moribund *P. nobilis* collected during a mortality event in 2017 were identified previously from 16S sequencing as *V. mediterranei* (Prado et al., 2020). Using previously described primers for the *atpA* gene (Lal et al., 2013) sequences were obtained from 16 strains isolated from moribund *P. nobilis*. BLAST analysis of the *atpA* sequences are in agreement with results obtained from 16S sequencing and show high identity values for the gene fragment isolated as originating from *V. mediterranei*. The *atpA* sequence was slightly more informative phylogenetically (Fig. 1). Phylogenetic analysis of the *atpA* sequences demonstrated the affiliation of the isolates from *P. nobilis* within the clade of *V. mediterranei*. Further, 7 of 16 *atpA* sequences obtained were 100% identical to the type strain (LMG 11258^{T}) used in a recent reevaluation of the *Vibrio* clades using MLST (Sawabe et al., 2013). There were 14 polymorphic sites among the *atpA* gene sequences and this is reflected in the distribution of strains within the *V. mediterranei* clade into two subclades.

The significance of genotypic differences among strains was further demonstrated by different antibiotic resistance phenotypes. The antibiograms of the 16 strains isolated in 2017-18 showed different sensitivity profiles, and sensitivities for the different antibiotics ranged most widely among strains for erythromycin (0- 28 mm diameter zone of inhibition among 16 strains) than for the other three tested antibiotics (florfenicol [30- 35 mm]; oxytetracycline [23- 34 mm]; norfloxicin [23- 31 mm]).

The main interest was pathogenicity of these strains, so primers for several vir genes were designed and used for obtaining additional information of the

genotypes of the strains (Tables 1 & 2). Screening of strains for virulence-related genes found some significant differences (Table 2.). Results for *zeta*, *ompU* and *rtx* exhibited significant variability among strains. All strains were positive for *sod* and *mshA*. Further, all strains examined were negative for *zot* and *toxR*. Whole genome sequencing of *V. shiloi/V. mediterranei* strains has shown that *mshA*, *rtx*, and *sod* are all significant determinants of virulence (Reshef et al., 2008). Based on the results of our genomic profiles we chose strain IRTA18-108 for the challenge experiment since it was positive for *rtx, mshA* and *sod*.

The *V. mediterranei atpA* primers from this study amplify a fragment of 914 bp, and a Sca I site was identified at approximately 384 bp from the 5´end of this *atpA* fragment. A search of *atpA* sequences from 54 other *Vibrio* spp. in GenBank identified several accessions for *V. mediterranei/shilonii*, all of which had a Sca I site. None of the other Vibrio species had a ScaI site in the *atpA* sequence. As a further confirmation of the identity of *V. mediterranei* by *atpA* amplification, a restriction fragment length polymorphism analysis using the enzyme Sca I was used. Only the amplified fragment from V. mediterranei was bisected into two smaller fragments of 530 bp and 384 bp (Fig. 2). All strains isolated from *P. nobilis* were further confirmed as *V. mediterranei* using this new *atpA* diagnostic assay.

In a trial comparing *V. mediterranei* genomic DNA with *V. anguillarum*, *V. alginolyticus, V. harveyi, V. splendidus*, and *V. aestuarianus*, amplification occurred only with *V. mediterranei* genomic DNA. Additionally, the assay demonstrated sufficient sensitivity to detect at least 56 fg of genomic DNA that is the equivalent of 10^0 CFU (Fig. 3). This result was also confirmed using viable plate counts. The limit of detection was reduced by one log when 1µg of host DNA was added to each mixture (limit of detection 10^1 CFU).

The animals used for the experimental challenge were measured to the nearest mm to establish the range of size of the collected animals, although they appeared likely to be from the same year class, thus of a similar level of immune maturation. Mean length was 23.97 ± 21.52 mm (SD) and the mean width was 107.19 ± 9.3 mm (SD). Animals above and below the mean were assigned to different tanks to avoid bias from all the small animals being placed together. The challenge experiment was initiated when water temperature was 20.1 ºC, but temperatures increased in the following days reaching a maximum of 24.5 ºC by 12 dpi. (Fig. 4). The first mortality was observed at 5 dpi in animals injected with 10^7 CFU, and when the water temperature had reached 22ºC. At 7 dpi the byssus had been released from 4 animals given an injection of 10^6 CFU´s; this is typically a sign of morbidity (unpublished observation). By 9 dpi there was 100% mortality in the tank containing animals injected with 10^5 CFU dose. When temperatures reached 24.5 ºC at 12 dpi, mortalities began to increase. From 13 dpi until the end of the experiment, temperature was steady at 24 ºC. This significant delay in the rise in temperature seemed to have some influence in the progression of cumulative mortalities from the various doses. Mortality was 100% by 15 dpi for the 10^7 CFU dose. By the end of the experiment at 23 dpi there was only 42.9% mortality from the 10^4 and the 10^6 CFU dose and 85.7% from the 10^3 CFU dose. At the end of the experiment, at 23 dpi, the relation between dosage and mortality was not linear.

Tissues dissected from animals that had died during the challenge experiment were analyzed by PCR and at least one tissue was positive for all injected animals. The tissue that was most commonly found to be positive was the adductor muscle. From three control animals injected with PBS and sacrificed

437 at the end of the experiment, all tissues tested negative. Histological analysis of
438 tissues from PCR positive animals exhibited some areas of infiltration of
439 haemocytes, suggestive of a localized inflammatory response, and some lesions
440 though no micro-colonies of bacteria in digestive tissue and kidney (data not
441 shown).

442 Selected animals that died after injection with 10^7, 10^5, and 10^3 CFUs, and
443 had been frozen were used for re-isolation of *V. mediterranei.* Kidney smears
444 inoculated onto TCBS agar resulted in abundant colonies of identical morphology
445 and yellow color, consistent with *V. mediterranei*. The Vm atpA PCR assay
446 confirmed identity of the bacteria as *V. mediterranei*.

447 Screening of DNA extracted from muscle tissue collected from moribund
448 animals during the 2017-18 mortality event indicated the presence of *V.*
449 *mediterranei* in 16/30 adductor muscle samples (Table 4). Intensity of
450 amplification suggests that the abundance of cells of *V. mediterranei* may have
451 diminished after treatment with the antibiotic florfenicol, however the timing of the
452 treatments also coincided with lower ambient winter water temperatures that also
453 likely played a significant role. *V. mediterranei* did persist in the animals after a
454 third florfenicol treatment and mortalities did not subside until all individuals were
455 transferred to a recirculation aquaculture system at 15ºC.

456

457 **Discussion**

458 It should be emphasized that working with a critically endangered protected
459 species at a time when populations are currently in steep decline due to infection
460 by *H. pinnae* (Catanese et al., 2018; López-Sanmartín et al., 2019) and various

other negative influences (climate change, habitat destruction, ocean acidification and eutrophication of coastal embayments, etc…) makes necessary certain compromises in experimental design. In this case, we used a limited number of animals (N= 7 /dose) and no replicates for each dose to gain the most information possible under the restrictions provided by the collection permit issued to our group. It was reasoned that if the bacteria is a true pathogen it should be able to kill at lower doses (10^3 CFU´s), as compared to killing at higher doses (10^7 CFU´s), which is more typical of opportunistic pathogens. The work was established with these parameters as guides, and the possibility of identifying the LD_{50} dose at the same time as demonstrating pathogenicity. Given that no control animals injected with sterile PBS died, the work does confirm the pathogenicity of the *V. mediterranei* strain IRTA18-108 in the host *P. nobilis*.

Since the identification of *P. nobilis* infected with *V. mediterranei* in 2018 (Prado et al. 2020), urgent questions have been raised about the distribution of this bacteria regionally and within captive rearing enclosures. Moreover, the identification of this bacterial species, associated to moribund or dead shellfish, does not by itself determine etiology. A challenge experiment is required to demonstrate that presence of this bacteria is necessary and sufficient to cause mortality. Further, a specific diagnostic is needed to confirm the presence of the bacteria in infected tissues, and to differentiate it from other species that would grow on semi-selective media such as TCBS. In a previous study of *V. harveyi*, the *atpA* gene was demonstrated to serve as a reliable marker for phylogenetic identification of multiple Vibrio species (Lal et al., 2013). The gene *atpA* encodes an enzyme that regulates the production of ATP from ADP in the presence of a transmembrane proton gradient. In this study, we identified short sequence motifs

within the *V. mediterranei atpA* gene that were sufficiently unique to design species-specific primers within the *atpA* open reading frame, thus obviating the need to use MLST for identification as recommended in other studies (Sawabe et al., 2013; Tarazona et al., 2014). The *V. mediterranei* specific PCR facilitates pathogen challenge work by providing the associated required bacterial identification without extensive sequencing or phenotypic assays.

While the *atpA* primers previously described (Lal et. al., 2013) amplified *atpA* fragments from all the species in our laboratory collection, there were additional non-specific bands (data not shown). Sequencing of the *V. mediterranei* strains in our collection and comparing to all other Vibrio *atpA* fragments found in GenBank showed that differentiation of *V. mediterranei* could be achieved using Sca I digestion. However, this RFLP assay was more cumbersome, and poorly amplified products would be even less visible after digestion. Therefore a more specific pair of primers was designed for amplification of *V. mediterranei atpA*. Specificity of the Vm atpA PCR diagnostic was demonstrated using genomic DNA from 5 Vibrio species present in our lab, while *in silico* analysis of an additional 54 distinct Vibrio *atpA* sequences indicated that these primers should function with high specificity. Primer design is of the utmost importance for surveying samples of a complex matrix (Wilcox et al., 2013). Filter-feeding aquatic animals represent a potential collection of a multitude of genomes mixed together creating a highly complex matrix for PCR diagnostics. We demonstrated the utility of this assay by using pure genomic DNA from bacteria spiked with 1µg of host DNA to demonstrate the assay still functions well. These *atpA* primers, alone, or together with Sca I digestion, serve as a clear identifier of *V. mediterranei*. Sensitivity was also tested using a serial dilution of

511 *V. mediterranei* DNA. This demonstrated that dilutions of DNA to 10^0 CFU
512 equivalents (56 fg) could be detected, and when spiked with 1 µg of host DNA
513 were detectable at 10^1 CFU equivalents.

514 Initial trials using the Vm atpA PCR to screen *P. nobilis* collected from Alfacs
515 Bay for a challenge experiment demonstrated 6/10 animals were contaminated
516 with *V. mediterranei*, but not in all tissues (baseline samples - Table 3). Only 1/10
517 were positive for *V. mediterranei* in the digestive gland, so the idea that this
518 bacteria is a common commensal of the digestive system seems unlikely.
519 Further, 4/10 were positive in the adductor muscle and 3/10 were positive in the
520 kidney; both tissues that are normally more likely to be nearly free of bacteria in
521 a healthy animal. However, with bivalves, the entirety of the internal organs are
522 fairly exposed to the aquatic environment and this can lead to surface
523 contamination of sampled tissues. Nevertheless, this should be equivalent for all
524 tissues. The positive signals from kidney and muscle are suggestive of
525 pathological processes, but more studies with in situ hybridization are needed to
526 follow up these findings.

527 The variability of results among doses of *V. mediterranei* inoculum could
528 have a variety of explanations. A slight difference in the tissue injected may have
529 influenced the progression of disease (valves were only partially opened to
530 administer the injections making specific positioning of the needle somewhat
531 difficult). Prior existence of other strains with different virulence profiles (some
532 animals tested prior to the challenge were already contaminated with some strain
533 of *V. mediterranei*) could have contributed to an effectively higher dose than was
534 calculated. Further, it has been shown that among strains there are both virulent
535 and non-virulent strains of this bacterial species (Reshef et al., 2008), highlighting

the need for correct species identification (see below). We chose to forego any prophylactic measures to depurate the *P. nobilis* of all *V. mediterranei* in this instance, since florfenicol seemed to be no guarantee of eradicating *V. mediterranei* (Prado et al., 2020). Also, there was a high risk of losing the limited number of animals of this protected species due to stress from captivity if the challenge experiment was not initiated somewhat quickly.

After the bacterial injection of the shellfish with *V. mediterranei* strain IRTA18-108, the aquaria were maintained as a closed system with daily water renewals. The ambient water temperature for the duration of the experiment was below the optimum described (25 ºC) for induction of coral bleaching by *V. shiloi* (Vattakaven et al., 2006). While temperatures remained below 22 ºC, the onset of mortalities was delayed, thus providing more opportunity for suppression of infection by the host immune response. As temperatures increased past 24 ºC all doses led to mortality (Figure 4) therefore, temperature would seem to be a predisposing factor, as reported previously in other host backgrounds (Kushmaro et al. 2001; Ben-Haim et al., 2002; Vattakaven et al., 2006).

The single strain used for inoculum in the challenge (IRTA 18-108) may not have been the most virulent among all strains isolated, however this strain was chosen based on it being among the most common 16S genotype among the 16 isolates for which we had sequence data, and positive for three significant virulence genes (Reshef et al., 2008). When a pathogen is not sufficiently virulent, its reproduction rate, and consequently transmission to a new host, are reduced (Ebert and Herre, 1996). Given that the 16S genotype we chose for the experimental challenge was common it suggested a high success in transmission. Moreover, non-virulent strains of this species are described

(Reshef et al. 2008). In nature, hosts are often infected by several strains with different pathogenic genotypes, which often leads to increased virulence (Read et al., 2001; Karvonen et al., 2012; Alizon et al., 2013; Susi et al., 2014). Additionally, *Vibrio* spp. are well known for their use of acyl-homoserine lactones as chemical signals for quorum sensing to get collaboration among multiple strains with different virulence factor profiles or different levels of expression of distinct virulence factors. Concerning the virulence genes identified, *sod* is understandably important for eliminating reactive oxygen species, which are toxic for the bacteria and produced by the host ROS response. The *rtx* gene encodes a cytolysin, which would inflict significant tissue damage on the host cells. Adhesin-like proteins, such as *mshA*, likely function in the scheme of virulence for attachment to mannose moieties on the surface of host cell membrane proteins (Ushijima et al., 2018). The strain IRTA18-108 was shown to have a combination of *vir* genes for distinct capabilities – attachment, cell lysis, and defense against host-produced reactive oxygen species – that may be sufficient for *V. mediterranei* to function as a pathogen in this host. More studies are needed to understand if *rtx*$^-$ strains are also pathogenic and if these genes are under temperature control as suggested by other studies of virulence in other hosts (Vattakaven et al., 2006; Rubio-Portillo et al., 2014; Serrano et al., 2018).

Since the pre-existence of *V. mediterranei* was documented in the animals collected for the challenge experiment, though the level of contamination among the animals used for challenge was not known, this played a significant role in the experimental outcome. Although the results did not show a linear relation between dose of injection and mortality rate, determination of pathogenicity was unequivocal as none of the control animals died. Mortality was not due to

586 temperature alone, since control animals were unaffected. Nor was mortality due
587 to presence of other pathogens, since the aspect of the colonies that appeared
588 on plates inoculated from frozen kidney samples suggest it was a pure culture,
589 and only *V. mediterranei* was identified from analysis by Vm atpA PCR post-
590 challenge.

591 The previous reports by Vattakaven et al. (2006) of VBNC in Vibrios were
592 found to be largely due to effects of starvation, with temperature being of
593 secondary importance. Thus freezing tissue samples of dead animals from the
594 challenge did not interfere with our ability to recover them in isolation for
595 confirming Koch´s postulates. From the first three animals from each tank that
596 died during the challenge, we tested tissues for presence of *V. mediterranei* using
597 the Vm atpA PCR. The injected animals were all positive in at least one tissue,
598 while the control PBS injected animals were negative. Although not all tissues
599 were positive among injected animals, the PCR results were consistent.
600 Moreover, Koch´s postulates were fulfilled since the strains of *V. mediterranei*
601 used for challenge were previously isolated from the kidney of the same host
602 species that had died.

603 Having demonstrated the efficacy of the Vmed atpA PCR, we wanted to
604 examine, retrospectively, samples of muscle tissues that had been collected
605 during the 2018 mortality episode (Prado et al., 2020). It was found that 53% of
606 the adductor muscle samples were positive for *V. mediterranei*. Among the 30
607 samples, 23% (Table 4) were strong positives (score of 3-5). However, these
608 samples included in equal numbers some that had died before and after
609 administration of florfenicol (Table 4). The intensity of positive PCR results
610 (although not strictly speaking quantitative) did seem to indicate that the intensity

of infection may have been reduced by antibiotic treatment. The incomplete elimination of *V. mediterranei* may be related to the dose or duration of treatment being insufficient, or that florfenicol is bacteriostatic rather than bacteriocidal in many bacteria (Papich, 2016). Another possible explanation may lie in the fact that different genotypes (based on *16S rDNA* and *atpA* sequencing, and vir gene PCR) and phenotypes (based on antibiograms) were observed, and some strains may have persisted due to different degrees of antibiotic sensitivity. Although the sensitivity for most strains was highest to florfenicol, additional strains may yet exist that are much more resistant.

Vibrio mediterranei/shiloi/shilonii has been described in recent publications as a pathogen of various aquatic animals (Rubio-Portillo et al., 2014; Serrano et al., 2018; Rodriguez et al. 2018), but as an emergent pathogen, surveillance tools are needed to be proactive about management of disease rather than reactive to epizootics. *V. mediterranei* is referred to by three different binomial names in various scientific literature. It is significant to clarify taxonomic nomenclature if emergent disease threats are to be recognized properly. Given a scenario of multiple diseases of equal severity, it is something more significant if one pathogen is affecting 6 host species, rather than six pathogens each affecting a single host species. In the case of *V. mediterranei,* to find hosts affected by this bacteria using keyword searching in the literature elicits different results depending on the specific epithet used (mediterranei, shilonii, or shiloi). As another example, in work by Reshef et al. (2008) they did a genomic subtractive hybridization of a pathogenic isolate of *V. shiloi* with a non-pathogenic species for the purpose of identification of genetic islands of pathogenicity specific to *V. shiloi*; the non-pathogenic species used as the “driver” for the subtractive

636 hybridization in this study was *V. mediterranei*. Clearly, development of
637 diagnostics need to be specific without confusion over nomenclature.

638 In summary, we demonstrate that *V. mediterranei* strain IRTA18-108 is
639 pathogenic in *P. nobilis,* and the genes *sod, rtx*, and *mshA* were implicated in the
640 virulence. During the challenge experiment no mortalities were observed until the
641 water temperature reached 22 ºC, and mortalities increased substantially after
642 the temperature surpassed 24 ºC validating earlier reports of a 24 - 25ºC
643 threshold for induction of virulence of *V. mediterranei* in other species
644 (Vattakaven et al., 2013; Serrano et al., 2018; Torres et al., 2018). Given that a
645 baseline sampling in this study found 60% of apparently healthy animals had a
646 basal contamination with this bacteria in at least one tissue, warming seawater
647 under climate change models can be expected to negatively impact remaining
648 populations of *P. nobilis* where *V. mediterranei* is endemic and water
649 temperatures exceed 24ºC. The new Vmed atpA PCR diagnostic developed for
650 this work should aid in managing other disease outbreaks where Vibrio spp. are
651 suspect.

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654 **Conflicts of Interest Statement**

655 The authors declare that they have no conflicts of interest.

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Ethics statement

The experiment complied with the Guiding Principles for Biomedical Research Involving Animals (EU2010/63), the guidelines of the Spanish laws (law 32/2007 and RD 53/2013), and authorized by the Ethical Committee of the Institute for Research and Technology in Food and Agriculture (Spain) for the use of laboratory animals.

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Figure 1. – The optimal tree from comparison of atpA gene sequences with the sum of branch length = -6881.5630 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. ● indicates *V. mediterranei* strains isolated from *P. nobilis*.

Figure 2. – Lanes 1-6 illustrate the specificity of *V. mediterranei* atpA PCR when primers are tested using genomic DNA of various related *Vibrio* species. M= molecular weight standard, an= *V. anguillarum*, al= *V. alginolyticus*, ha= *V. harveyi*, sp= *V. splendidus*, me = *V. mediterranei*, ntc= no template control, ScaI= *V. mediterranei* amplicon digested with Sca I restriction enzyme.

Figure 3. – Serial dilutions of DNA from 56 ng to 56 fg were used for testing the sensitivity of the *V. mediterranei* atpA PCR. The 10^0 sample (56 fg) would equal 1- 10 cells according to plate counts. C- = no template control.

Figure 4. – Results of a bacterial challenge experiment over a 23-day time course showing percent cumulative mortality in *P. nobilis* after injection with different doses of *V. mediterranei* strain IRTA 18-108. Daily water temperature is shown on secondary y-axis.

Table 1. Primers used in this study for amplification and sequencing.

Target		Sequence (5´-3´) Forward		Reverse	Amplicon Size (bp)	Source
16S rDNA	VHAAF1	GGTATCATCCGCATCCACGG	VHAAR1	CTTTTGCGCCGGCCATCG	1500	Lal et al. 2013
16S rDNA *V. mediterranei*	Vib-atpA-F	CAATTGAAGCTAAACTTACGTC	Vib-atpA-R	CCGTGGCTTAGCTGACGCTTAG	914	this study
major subuniit for msh A pilli	Vm mshA-F	ATGTCGAAGCGTGCGTATTCC	Vm mshA-R	GGTACGAATATCGTACTCGAAG	179	this study
outer membrane protein U	ompU-F	ATATTAGCGGCACCGTGGATCAGG	ompU-R	GCTGAAACGCCGCCGCCAGACG	308	this study
rtx toxin	Vm rtx-F	GATACCGTCACCAGCACAGCATC	Vm rtx-R	CATAAGTCAAATTGCCATTAGGAACG	297	this study
superoxide dismutase	Vm sod-F	CCAGTTAACTAGAGCCCAG	Vm sod-R	TGACGGTTCTCTAGATATCGTG	165	this study
zonular occludins toxin	Vm zot-F	GTGTTAACAGAGTGATGTCGATACC	Vm zot-R	GTTTCCGAACTCATCGAGGTTAAG	231	this study
toxin R	VmtxR-F	TAGCAGCCTGACCCAGGCGATTAG	VmtxR-R	TAAAGCTGCTCTGCGCCGGGTTGG	355	this study
zeta toxin (toxin P)	Zeta-F	CTCAAGAAGAGTCTGCGGTGTCGG	Zeta-R	CGACCACGCTTTAATGAGCGCTGG	318	this study

Table 2. Virulence gene profile of the 16 studied strains of *V. meditteranei*. Asterix marks the strain used in the challenge experiment.

Vibrio mediterranei strain ID	Virulence Genes *mshA*	*ompU*	*rtx*	*sod*	*zot*	*toxR*	*zeta*
IRTA 18-94	+	+	+	+	-	-	-
IRTA 18-95	+	-	-	+	-	-	-
IRTA 18-97	+	-	-	+	-	-	-
IRTA 18-98	+	-	+	+	-	-	+
IRTA 18-99	+	-	-	+	-	-	-
IRTA 18-100	+	-	+	+	-	-	+
IRTA 18-102	+	-	+	+	-	-	+
IRTA 18-103	+	+	+	+	-	-	-
IRTA 18-104	+	-	+	+	-	-	-
IRTA 18-105	+	+	-	+	-	-	-
IRTA 18-107	+	-	-	+	-	-	-
IRTA 18-108*	+	-	+	+	-	-	-
IRTA 18-109	+	-	+	+	-	-	-
IRTA 18-110	+	-	+	+	-	-	-
IRTA 18-111	+	-	+	+	-	-	-
IRTA 18-112	+	-	+	+	-	-	-

Table 3. PCR results from testing *P. nobilis* tissues before and after challenge with different doses of *V. mediterranei* strain IRTA 18-108. An arbitrary score (0-

845 5) is provided to indicate the intensity of amplification as a proxy for actual
846 quantitative PCR results.

847

Sample	Treatment	Adductor muscle	Branchia	Gonad	Kidney	Digestive gland
1	baseline	1	0	0	0	0
2	baseline	0	0	0	0	0
3	baseline	2	0	0	1	1
4	baseline	1	0	0	0	0
5	baseline	0	0	1	0	0
6	baseline	0	0	0	0	0
7	baseline	2	2	0	1	0
8	baseline	0	0	0	0	0
9	baseline	0	0	0	1	1
10	baseline	0	0	0	0	0
C-1	control	0	0	0	0	0
C-2	control	0	0	0	0	0
C-3	control	0	0	0	0	0
3-1	10^3 CFU	5	3	3	0	0
3-2	10^3 CFU	4	0	1	0	0
3-3	10^3 CFU	5	1	2	0	1
4-1	10^4 CFU	4	1	4	1	0
4-2	10^4 CFU	3	0	0	2	1
4-3	10^4 CFU	0	0	0	0	2

5-1	10^5 CFU	5	2	4	0	0
5-2	10^5 CFU	2	1	3	0	0
5-3	10^5 CFU	5	0	4	0	0
6-1	10^6 CFU	4	0	2	2	0
6-2	10^6 CFU	0	0	1	0	0
6-3	10^6 CFU	3	0	1	1	2
7-1	10^7 CFU	2	0	2	0	0
7-2	10^7 CFU	1	0	0	0	0
7-3	10^7 CFU	4	0	5	1	0

Table 4. PCR results from representative samples of adductor muscle tissue collected before and after florfenicol treatment during an extended period of mortality from 2018-19. Intensity of PCR amplifications were assigned an arbitrary score of 0 (negative) to 5 (most intense positive). Date of death, water temperature, and florfenicol treatment are recorded.

Sample	Date of death	Temp (ºC)	Florfenicol treatment	V. med PCR (scale 0 - 5)
1	30/04/2018	16.1	-	**5**
2	15/05/2018	18.4	-	**0**
3	15/06/2018	22.7	-	**0**
4	22/06/2018	23.5	-	**0**
5	25/06/2018	23.5	-	**5**

6	14/08/2018	26.1	-	**1**
7	16/08/2018	25.9	-	**0**
8	16/08/2018	25.9	-	**1**
9	30/08/2018	25.4	-	**1**
10	31/08/2018	25.4	-	**0**
11	03/10/2018	18.4	-	**5**
12	09/10/2018	18.3	-	**0**
13	12/10/2018	18.3	-	**3**
14	12/10/2018	18.3	-	**5**
15	19/10/2018	18.9	-	**0**
16	03/12/2018	15.6	1º FFC	**0**
17	03/12/2018	15.6	1º FFC	**3**
18	17/12/2018	13.8	1º FFC	**1**
19	18/12/2018	13.8	1º FFC	**0**
20	31/12/2018	12.9	1º FFC	**0**
21	02/01/2019	12.9	1º FFC	**0**
22	11/02/2019	12.6	2º FFC	**0**
23	22/02/2019	13.9	2º FFC	**1**
24	11/03/2019	16.9	2º FFC	**1**
25	15/04/2019	17.5	2º FFC	**2**
26	06/05/2019	18.2	3º FFC	**1**
27	06/05/2019	18.2	3º FFC	**1**
28	07/05/2019	19.1	3º FFC	**0**
29	09/05/2019	19.1	3º FFC	**0**
30	27/05/2019	19.8	3º FFC	**4**

857

858

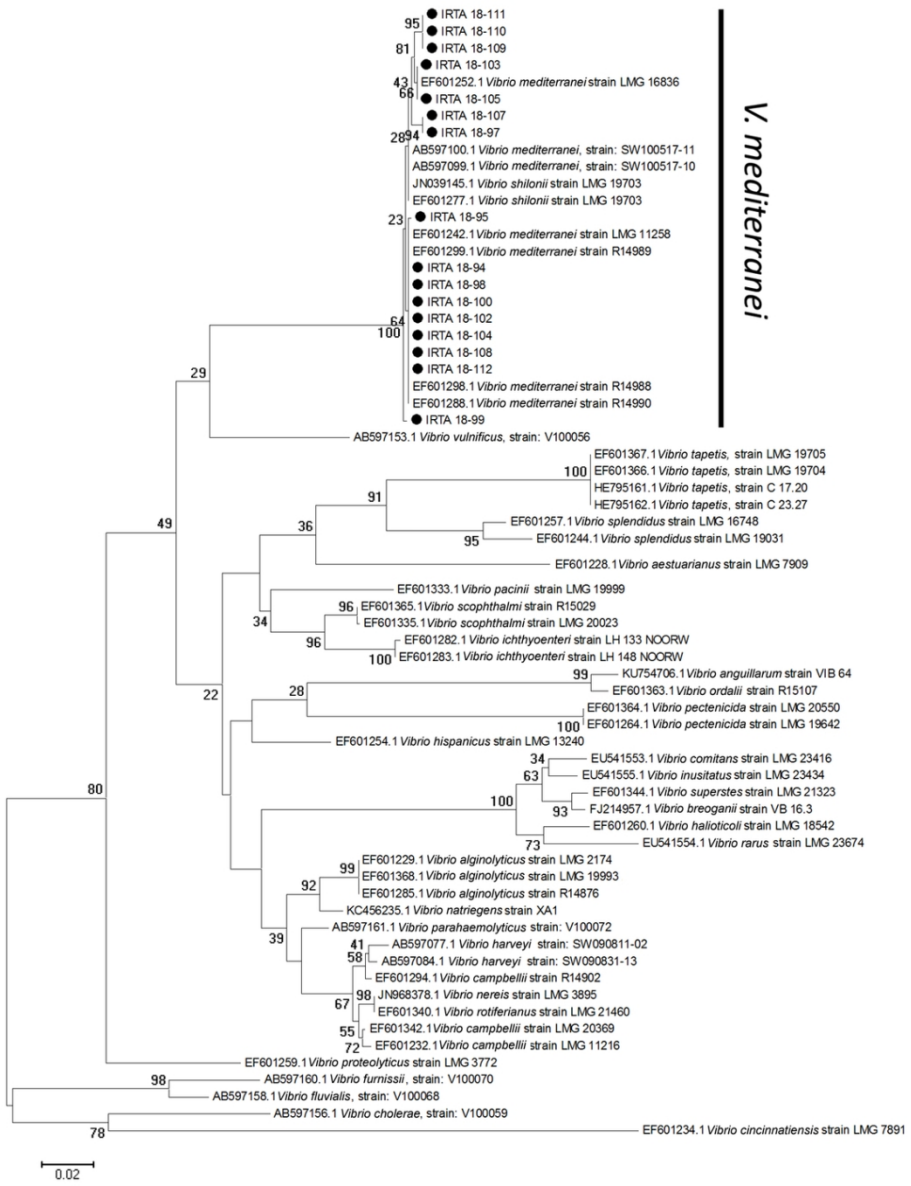

Figure 1. – The optimal tree from comparison of atpA gene sequences with the sum of branch length = -6881.5630 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. ☐ indicates V. mediterranei strains isolated from P. nobilis.

97x122mm (300 x 300 DPI)

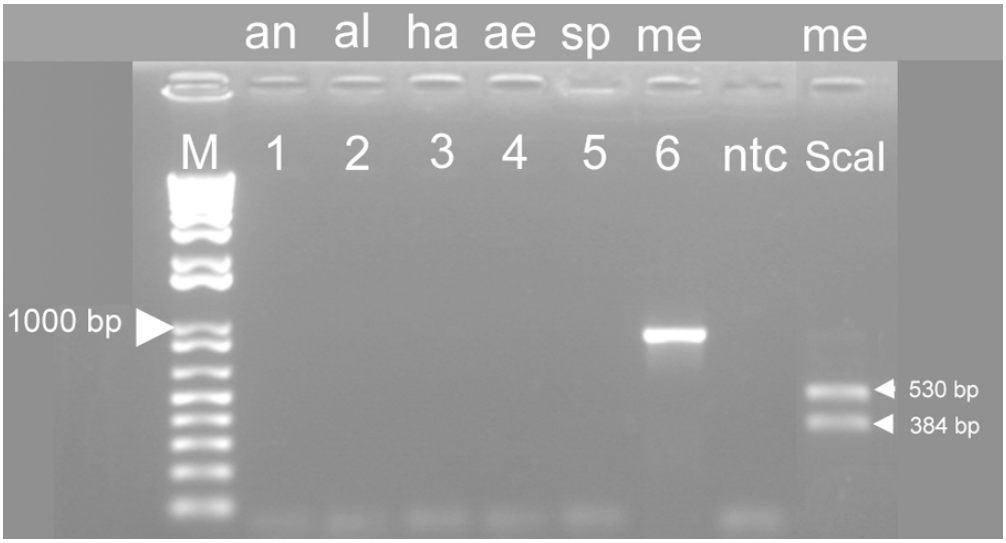

Figure 2. – Lanes 1-6 illustrate the specificity of V. mediterranei atpA PCR when primers are tested using genomic DNA of various related Vibrio species. M= molecular weight standard, an= V. anguillarum, al= V. alginolyticus, ha= V. harveyi, sp= V. splendidus, me = V. mediterranei, ntc= no template control, ScaI= V. mediterranei amplicon digested with Sca I restriction enzyme.

76x40mm (300 x 300 DPI)

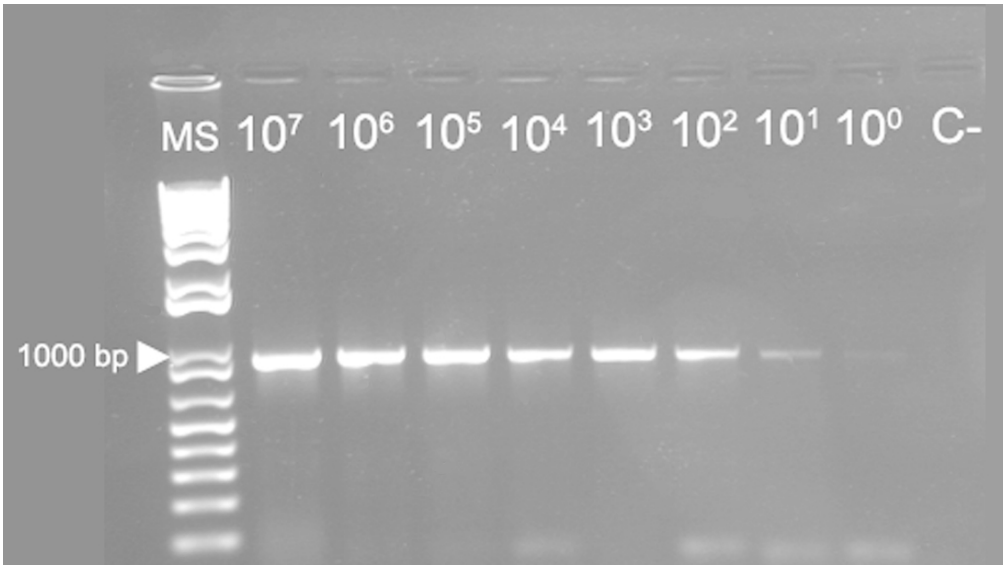

Figure 3. – Serial dilutions of DNA from 56 ng to 56 fg were used for testing the sensitivity of the V. mediterranei atpA PCR. The 100 sample (56 fg) would equal 1- 10 cells according to plate counts. C- = no template control.

92x51mm (300 x 300 DPI)

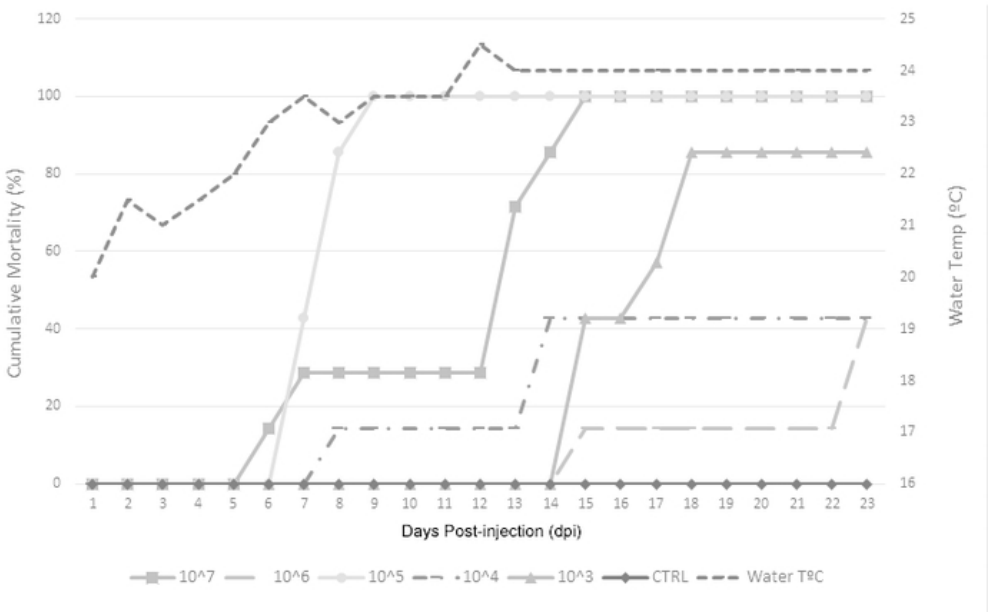

Figure 4. – Results of a bacterial challenge experiment over a 23-day time course showing percent cumulative mortality in P. nobilis after injection with different doses of V. mediterranei strain IRTA 18-108. Daily water temperature is shown on secondary y-axis.

59x37mm (300 x 300 DPI)