First description outside Europe of the emergent pathogen *Vibrio europaeus* in shellfish aquaculture

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DOI: 10.1016/J.JIP.2021.107542

https://www.sciencedirect.com/science/article/abs/pii/S0022201121000094?via%3Dihub

Journal: *Journal of Invertebrate Pathology (short communication)*

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Abstract

Vibrio europaeus is an emergent pathogen affecting the most important bivalve species reared in Spanish and French hatcheries. Using a genomic approach, we identified *V. europaeus* outside Europe for the first time from massive larval mortalities of scallop (*Argopecten purpuratus)* in Chile and from seawater near a shellfish hatchery in the US West Coast. Results show the worldwide spreading and potential impact of *V. europaeus* for aquaculture; these four countries are among the 10 major producers of mollusks. Pathogenicity of *V. europaeus* was demonstrated for the first time towards scallop, the second most important species for Chilean mariculture.

Keywords: *Vibrio*, virulence, vibriosis, shellfish aquaculture, scallop larvae, hatchery.

Manuscript

Hatcheries constitute an important resource to provide the high number of seed required by shellfish farmers because seed recruitment in wild beds has decreased in recent years. Various factors are involved, such as overfishing, climate change and diseases (da Costa et al., 2020). Vibriosis, caused by some *Vibrio* species, is the most important bacterial disease affecting the bivalve production process in hatcheries (Dubert et al., 2017a). During recent years *V. europaeus* has been responsible for mass mortalities in outbreaks that affected different shellfish hatcheries in France and Spain (Dubert et al., 2016, 2017b; Mersni-Achour et al., 2014, 2015; Prado et al., 2005, 2015; Saulnier et al., 2010; Travers et al., 2014).

Pathogenicity of *V. europaeus* has been demonstrated by experimental infections over a wide host range. The most important bivalve species reared in the European hatcheries are affected, including Pacific oyster (*Crassostreae gigas)* and Manila clam (*Ruditapes philippinarum),* ranked in the top 10 major species produced in world aquaculture (FAO, 2018). *V. europaeus* is pathogenic to different stages of the life cycle of bivalves such as larvae of oysters (*C. gigas*

and *Ostrea edulis*) and clams (*R. decussatus, R. philippinarum, Donax trunculus, Ensis arcuatus* and *Polititapes romboides*), early spat in Manila clam (*R. philippinarum*) and later spat (juveniles) in Pacific oyster (*C. gigas*) (Dubert et al., 2016, 2017b; Mersni-Achour et al., 2014, 2015; Prado et al. 2005, 2015; Travers et al., 2014). *V. europaeus* has been also associated with mortalities in important cultured gastropods such as abalones (*Haliotis* spp.) (Saulnier et al., 2010).

In this study, we characterised two bacterial strains isolated from aquaculture environments in Chile and United States, NPI-1 and 071316F, respectively, and the potential impact for shellfish aquaculture was evaluated. The Chilean strain was associated with massive mortality of scallop larvae (*Argopecten purpuratus*) and we demonstrated its virulence in the original host by experimental infections.

NPI-1 was isolated as a predominant strain during an episodic outbreak of disease in which farmers detected high mortalities (70%) in larval cultures of *A. purpuratus* (veliger larvae; 10 days old; 126,80 ± 4,42 µm). The larvae were reared in conical tanks (8000 L) at 18ºC in a commercial hatchery located in Tongoy Bay (Chile) (30°15′27″S 71°29′33″W). Microbiological sampling and bacterial isolation were performed following Rojas et al. (2019). The genome of the strain NPI-1 was sequenced by SNPsaurus using PacBio Sequel II (Pacific Biosciences) and HiSeq 4000 (Illumina) sequencers. Quality control of Illumina reads was performed using Trimmomatic (Bolger et al., 2004), filtering poor quality reads and trimming poor quality bases. Assembly of PacBio reads was performed using de novo long-read assembler Flye (Kolmogorov et al., 2019) and polished by Racon (Vaser et al., 2017). Finally, the resulting assembly was curated by the Illumina reads using Pilon tool (Walker et al., 2014).

In a previous study, Gradoville et al. (2018) reported that Netarts Bay (Oregon, United States) (45°24'52"N 123°56'05"W) had a high prevalence of oyster-pathogenic *Vibrio* spp., such as *V. coralliilyticus,* especially during the summer and low tide. Strain 071316F was isolated in Netarts Bay in July 2016 from seawater near a shellfish hatchery following the procedures

described by Kehlet-Delgado et al. (2020) for bacterial isolation. Assembly of the draft genome was obtained from Miseq (Illumina) sequencing. Final genome assemblies were annotated with Rapid Annotations using Subsystems Technology (RAST Server) (Aziz et al., 2008) and deposited at DDBJ/EMBL/GenBank (Table S1).

Workflow described by Chun et al. (2018) was followed to classify strains NPI-1 and 071316F at the species level. First, the 16S rDNA sequence was retrieved from genome annotation and used in a 16S-based search on EzBioCloud database (Yoon et al., 2017). Average Nucleotide Identity (ANI) calculations were performed using OrthoANI algorithm and digital DNA-DNA hybridization (dDDH) genomes by means of the Genome-to-Genome Distance Calculator using Formula 2 (identities/HSP length) (Meier-Kolthoff et al., 2013; Lee et al., 2016). An up-to-date bacterial core gene set (UBCG) was used to infer phylogenomic relationship between strains NPI-1 and 071316F and the related taxa using a set of 92 core genes (Na et al., 2018). The pipeline uses Prodigal and hmmsearch for gene finding and identification from genome assemblies, respectively (Eddy, 2011; Hyatt et al., 2010). The individual UBCGs were aligned, concatenated and alignment positions showing more than 50% gap characters were excluded. The final nucleotide alignments were used to infer the maximum likelihood (ML) phylogenetic trees using FastTree with the GTR model by bootstrapping over 1000 replications and Gene Support Indices (GSI) (Price et al., 2010). The phylogenetic tree generated from the concatenated alignment of 92 UBCGs was visualized and edited with MEGA7 (Tamura et al., 2013). On the basis of 16S rDNA sequences, *V. europaeus* and *V. bivalvicida*, species belonging to the Orientalis clade, were the closest relatives to NPI-1 (99.93% and 98.98%, respectively) and 071316F (100% and 99.00%, respectively) (Table S2). Remaining results were below the 98.70% cut-off proposed to delineate bacterial species by Stackebrandt and Ebers (2006) (Table S2). The Orientalis clade has relevant significance for bivalve aquaculture because it includes well-known bivalve pathogens such as *V. europaeus, V. bivalvicida* and *V. tubiashii* (Dubert et al., 2017a).

For genomic analyses, we also sequenced the genome of *V. europaeus* 07/118 T2 (=CECT 8426), a strain responsible of massive mortalities of *C. gigas* spat in a French hatchery (Travers et al., 2014). We followed the same procedure described for NPI-1 and obtained a fully resolved genome for *V. europaeus* (Table S1). Genomic comparisons (Table S3) showed that only percentages of OrthoANI (>98%) and dDDH (>84%) compared with NPI-1 or 071316F and *V. europaeus* were higher than the species-delineating thresholds established for ANI (95~96%) and for dDDH (70%) (Meier-Kolthoff et al., 2013; Richter and Rosselló-Móra, 2009). Genome-based phylogeny (Figure 1), according with OrthoANI and dDDH results, strongly supported (100 bootstrap) the taxonomic affiliation of strains NPI-1 and 071316F with *V. europaeus* (Figure 1). For strain 071316F, GSI showed that 79 out of 92 UBCGs supported this concatenated alignment and suggested that possible events of lateral gene transfer occurred in twelve genes (Na et al., 2018). In addition, results based on ANI, dDDH and phylogenomics indicated that the French strain (07/118 T2) was the closest relative to NPI-1 (Figure 1 and Table S3).

Virulence of the strain *V. europaeus* NPI-1 was evaluated in its original host by experimental infections as previously described Rojas et al. (2019). Challenges were performed in 12-well microplates for 60 h (18ºC, dark) and in triplicate (Orange Scientific). Healthy *A. purpuratus* larvae (122.56±4.86 µm) were inoculated at a final concentration of 1.0×10^5 CFU mL^{-1} and wells without bacteria were included as negative controls in all experiments. In addition, bacterial concentration required to kill the 50% of infected scallop larvae (LD_{50}) was calculated with R by fitting the mortality curves to a logistic regression model using MASS package (Venables & Ripley, 2002) and visualized with ggplot2 package (Wickham, 2016). Larvae were checked under inverted microscopy (Nikon Eclipse Ts2) and most of them showed the typical signs of vibriosis after 24 h: erratic swimming was the first clinical sign followed by disruption of the velum, necrosis of the digestive gland and bacterial swarming. NPI-1 infection led to high mortality rates (90.03±6.75%) at 48 h and reached the 95.79±5.95% at the end of the

experiment in all replicates. Mortalities in the negative controls were lower than 4.61±1.34% (Figure 2A). According to Koch´s postulates, strains were re-isolated from inoculated wells after each challenge. Virulence of the strain NPI-1 was estimated by its LD_{50}: 1.16×10^5 CFU mL^{-1} at 24 h and 1.59×10^2 CFU mL^{-1} at 48 h (Figure S1). Interestingly, strain NPI-1 was less virulent than the strain *V. europaeus* 07/118 T2, which produced an LD_{50} value of 2.3×10^3 CFU ml^{-1} after 24 h post-infection towards its original host (*C. gigas* larvae) (Mersni-Achour et al., 2015).

Finally, the role of the extracellular products (ECP) released by the strain *V. europaeus* NPI-1 in its original host was also evaluated using the protocol described by Rojas et al. (2019) based on cellophane plate technique. Experimental infections were performed as described above and healthy scallop larvae (125.6 ± 4.84 µm) were inoculated with ECP-cell free supernatant to obtain a final protein concentration of 2, 4 and 8 µg mL^{-1}. Percentage of mortalities were directly related with the ECP concentrations and were 32.86±4.27%, 46.30±4.52% and 62.50±6.22% at 60 h, respectively (Figure 2B). As previously observed by Mersni-Achour et al. (2015) for the virulent strain *V. europaeus* 07/118 T2, even the most enriched fraction of ECP was less toxic to larvae than whole bacterial cells, suggesting that multiple virulence factors are required by *V. europaeus* to induce a complete infection phenotype.

Our results demonstrated the global spread of the emergent pathogen *V. europaeus*. This is particularly relevant for shellfish aquaculture because *V. europaeus* has been detected in four of the ten major world producers of marine mollusks, including Spain (#6) and France (#9), and now for the first time Chile (#4) and the US (#8) (FAO, 2018). We also showed that the *V. europaeus* host range includes *A. purpuratus*, the second most important species for Chilean aquaculture.

Conflict of interest

All the authors declare that they have no conflicts of interest.

Acknowledgments

This study was supported by the Strategic Researcher Cluster BioReDeS funded by the Regional Government Xunta de Galicia (ED431E 2018/09), by a postdoctoral fellowship from Xunta de Galicia (ED481B 2016/032) and, in part, AGL2017-86183-R (Ministerio de Ciencia e Innovación, Spain).

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FIGURES

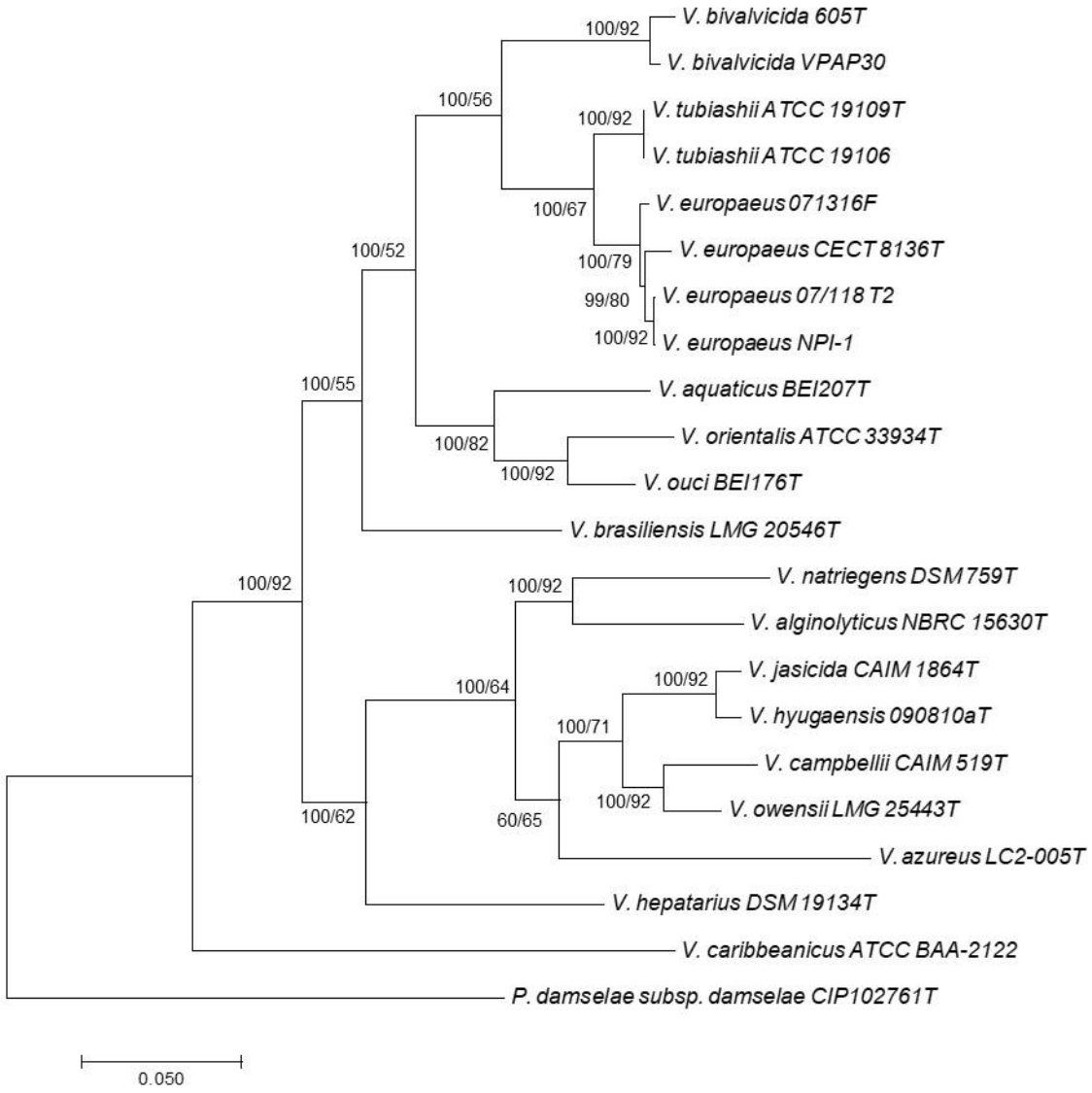
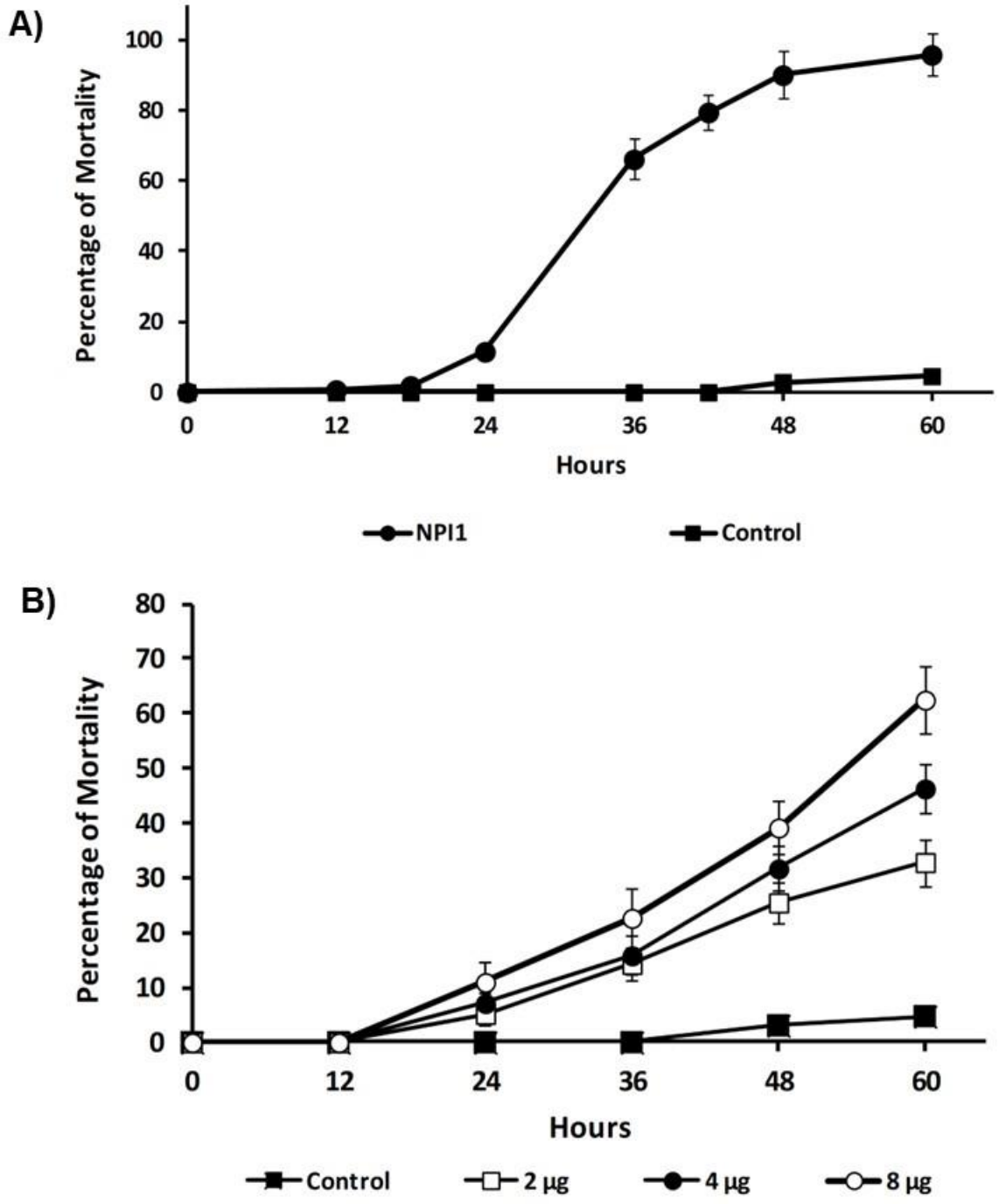


Figure 1. ML phylogenetic tree using GTR model inferred from UBCGs between strains NPI-1 and 071316F and their related taxa.

Photobacyerium damselae subsp. damselae CIP102761^{T} (Assembly accession: GCA_000176795.1) was used as the outgroup. The length of concatenated alignment was 88,779 bp. Bootstrap values and GSI percentages are indicated at branching points separated respectively by a slash. Bar, 0.050 substitution per position.



288

289 **Figure 2. Mortalities (%) of scallop (*Argopecten purpuratus*) larvae after NPI-1 infection**

290 **using whole cells (A) and ECPs (B) at different protein concentrations.**

291 **Values are a mean ± SD of three replicates**.