Vibrio aestuarianus detection by Real Time Polymerase Chain Reaction

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Ifremer, Laboratoire de Génétique et Pathologie des Mollusques Marins, Av. de Mus de Loup, 17390 La Tremblade, France

Vibrio aestuarianus detection by Real Time Polymerase Chain Reaction

1. Scope

This procedure explains a standard diagnostic test used for the detection of the bacteria *Vibrio aestuarianus* in bivalves. It allows detecting all the strains of *V. aestuarianus* known until now (*subspecies francensis, aestuarianus and cardii)* based on the amplification of a fragment of the *dnaJ* gene encoding heat shock protein 40 (GenBank # AB263018).

2. References

Garcia, C., Mesnil, A., Tourbiez, D., Moussa, M., Dubreuil, C., Gonçalves de Sa, A., ... & Travers, M. A. 2021. *Vibrio aestuarianus* subsp. *cardii* subsp. nov., pathogenic to the edible cockles *Cerastoderma edule* in France, and establishment of *Vibrio aestuarianus* subsp. *aestuarianus* subsp. nov. and *Vibrio aestuarianus* subsp. *francensis* subsp. nov. *International Journal of Systematic and Evolutionary Microbiology*, *71*(2), 004654.

Saulnier D., De Decker S, Haffner P., 2009. Real-time PCR assay for rapid detection and quantification of *Vibrio aestuarianus* in oyster and seawater: a useful tool for epidemiologic studies. *Journal of Microbiological Methods* 77(2), 191-197

Garnier M, Labreuche Y, Nicolas JL, 2008. Molecular and phenotypic characterization of *Vibrio aestuarianus* subsp francensis subsp nov., a pathogen of the oyster *Crassostrea gigas*. *Systematic and Applied Microbiology* 31(5), 358-365.

Nhung PH, Shah MM, Ohkusu K, Noda M, Hata H, Sun XS, Iihara H, Goto K, Masaki T, Miyasaka J, Ezaki T, 2007. The *dna*J gene as a novel phylogenetic marker for identification of *Vibrio* species. *Systematic and Applied Microbiology,* 30(4), 309-315.

Tison DL, Seidler RJ, 1983. *Vibrio aestuarianus* - a new species from estuarine waters and shellfish. *International Journal of Systematic Bacteriology* 33(4), 699-702.

3. Equipment and environmental conditions

This test requires the equipment and environmental conditions classically used for Real Time PCR assays:

- A closed hood equipped with an UV producing system to eliminate potential contaminations when preparing PCR mix,
- Two complete sets of pipettes (2 µL; 20 µL; 200 µL and 1000 µL), the first one for DNA extraction, and the second one for PCR mix preparation,
- An additional pipette (20 µL) to dispense samples in PCR mix,
- Filter pipette tips (2 µL; 20 µL; 200 µL and 1000 µL) for DNA extraction, PCR mix preparation and sample dispensing,
- Real Time PCR plates or PCR tubes
- A thermocycler for real-time PCR

Manipulator must wear a lab coat and gloves during all the different steps described below. Lab coat and gloves must be changed preferably after each main step: DNA extraction, preparation of PCR mix, sample dispensing and amplification.
It is recommended to perform these different steps in different rooms. Amplification particularly should take place in a room separate from DNA extraction, PCR mix preparation and DNA dispensing.

4. Procedure

4.1. Sample preparation

DNA can be extracted from a representative colony selected after bacteria isolation or from bivalve tissues.

4.1.1. DNA extraction from a representative colony

Predominant bacteria are isolated from marine mollusc tissues according to the SOP "Isolation of predominant bacteria in marine molluscs" available on the EURL website : https://www.eurl-mollusc.eu/SOPs.

Bacteria corresponding to one representative colony are sampled with a wooden sterile toothpick and put in suspension into two hundred (200) µl of molecular biology grade water. Total DNA is extracted by heating the samples for ten minutes at 98°C. After a quick cooling at 4°C, five µl are immediately used for the assay.
Note: too much bacteria in the suspension may inhibit the PCR reaction.

4.1.2. DNA extraction from bivalve tissues

Live or freshly dead (not decaying) bivalves, which can be previously frozen or stored in ethanol, are processed for DNA extraction.
Samples are processed differently according to their size:

a) For larvae or spat smaller than 6mm, **pools** of 50 mg of whole animals (remove the shell when possible in order to avoid PCR inhibition) completed with 200 µL of distilled water are crushed and centrifuged at 1000 g for 1 minute.
b) For spat between 6 and 15 mm in size, all the soft tissues from each animal are grinded **individually**.
c) For animals bigger than 15 mm, pieces of **gills, mantle and digestive gland** are sampled and processed **individually**.

DNA extraction can be performed for example using the Wizard Genomic DNA Purification Kit from Promega®, or the QIAamp® DNA Mini Kit from QIAGEN ®, and following the instructions for Tissue Test Protocol.
Note: burrowing clams such as cockles may contain PCR inhibitors, other extraction kits may be used to reduce the presence of inhibitors.
It is recommended to check the quality and efficacy of the extraction by measuring the optical density (260 nm) with a spectrophotometer, or by electrophoresis in agarose gel.
DNA solutions are kept at 4°C +/- 3°C until PCR analyses are performed.

4.2. Real Time Polymerase Chain Reaction

4.2.1. Reactives

MasterMix for Taqman® PCR, for example : BioRad SsoAdvanced Universal Probes Supermix #1725280, Brilliant III Ultra-Fast QPCR Master Mix (Agilent Technologies® ref: # 600881)…

H_20 (free of DNA and RNA)

4.2.2. Primers and Probes

*dna*J-F	5' GTATGAAATTTTAACTGACCCACAA 3'
*dna*J-R	5' TCAATTTCTTTCGAACAACCAC 3'
*dna*J -probe	5' HEX TGGTAGCGCAGACTTCGGCGAC BHQ-1 3'

<u>Note</u>: Primers amplify a PCR product of 267 bp.

4.2.3. PCR Mix

PCR mix for each tube is:

	Initial concentration	**Volume per tube**	**Final concentration**
2X Master Mix	2X	10µL	1X
*dna*J-F	20 µM	0.3µL	0.3µM
*dna*J-R	20 µM	0.3µL	0.3µM
*dna*J -probe (HEX)	10 µM	0.4µL	0.2µM
H_2O		4µL	

15 µL of this PCR mix is dispensed in each Real Time PCR plate well
5 µL of extracted DNA (diluted 1/10 or adjusted at 5 ng/µL) is added to each tube

Each sample can be tested in simplicate or duplicate
Real-time PCR mix should be prepared in excess (for example for two additional samples)

4.2.4. Controls

Negative controls aim at detecting potential cross contamination of working environment during the DNA extraction and the PCR. At least two negative controls should be included for each test: one extraction negative control (could be a known negative sample or water extracted at the same time as the samples) and one PCR negative control (could be water added in the PCR mix instead of the DNA sample).
Positive controls allow verifying that PCR assays have performed correctly. Positive controls consist of DNA extracted from oyster known to be infected with *V. aestuarianus,* or DNA extracted from a *V. aestuarianus* strain, or plasmidic DNA containing the PCR target. At least one positive control should be included for each PCR run.

4.2.5. Standard curves (optional, for quantitative assays only)

Standard curves are prepared using dilutions of bacterial or plasmidic DNA suspension.
This suspension corresponds to a known amount of bacterial DNA copies (extracted from bacteria culture) or to a known amount of plasmidic DNA including the target region *dna*J - F- *dna*J - R.

This suspension is 1:10 serially diluted. The standard curve should include at least 6 dilutions:

Copies of *Vibrio aestuarianus* DNA/µL in the initial DNA suspension
1^{E8}
1^{E7}
1^{E6}
1^{E5}
1^{E4}
1^{E3}

Standard curves aim at checking the efficacy of the PCR reaction and at estimating the amount of bacteria copies present in tested samples.
One standard curve should be included for each quantitative real-time PCR analysis.

4.2.6. Amplification

Amplification cycles are performed using a thermocycler for real-time PCR (for example CFX from BioRad®or Mx3000 Thermocycler sequence detector from Stratagene®).

-Initial denaturation: **3 min* at 95°C**
-Amplification: **40 cycles (10 sec at 95°C and 20 sec at 60°C)**

* The initial denaturation time may vary depending on the Master mix used

The fluorescence is recorded at the end of each cycle with HEX filter.

4.2.7. Interpretation

Threshold cycle (C_t) is defined as the cycle at which a statistically significant increase in fluorescence output above background is detected. Cts are calculated automatically by the thermocycler software. If samples were tested in duplicates, reported Ct values are calculated as averages of both replicates for each reaction.

Controls

Before concluding about the status of the tested samples regarding the presence of *V. aestuarianus*, results obtained with control samples should be checked. A PCR run is valid if negative controls do not present any amplification and positive controls present amplification.

Samples

A sample is considered positive when a characteristic amplification curve is observed.

A sample is considered negative when there is no amplification curves.

Depending on the context / objective of the analysis, a cut-off Ct value can be used to define if samples are positives or negatives. For example, when analysing DNA extracted from bacterial colony (section 4.1.1), the PCR signal should be strong if this colony correspond to *V. aestuarianus*. In case of Ct>30 on such sample, we recommend performing sequencing to confirm the detection.

In case of quantitative assays: the efficiency of the standard curve should be between 90-110%, the slope of the curve should be -3,3 +/- 0,5, and R^2 should be at least 0,98.