Bonamia sp. detection and characterization by *in situ* hybridization (ISH)

Content

Editions

Edition	Date	Updated part
N° 1	May 2013	Creation
N° 2	December 2024	Creation of a general SOP for *in situ* hybridization connected with diseases-specific SOPs. This SOP was reviewed to standardise the content of diseases-specific SOPs, to include new ISH probes for the detection of *B. ostreae* and *B. exitiosa* and to add information regarding the specificity of ISH probes

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1. Scope

This procedure describes *In Situ* Hybridization (ISH) assays for the detection of *Bonamia* sp., *B. ostreae* and *B. exitiosa*.
ISH assays are used to confirm a previous histological diagnosis at the genus level and/or at the species level.

General steps to perform ISH assays are described in the SOP "Detection of mollusc pathogens by *In Situ* Hybridization (ISH)

This SOP provide additional information on the probes to be used for the specific detection of *Bonamia* parasites and result interpretation.

2. References

Cochennec, N., Le Roux, F., Berthe, F., and Gerard, A. **(**2000). Detection of *Bonamia ostreae* based on small subunit ribosomal probe. Journal of Invertebrate Pathology 76(1): 26-32.

Hill, K. M., Carnegie, R. B., Aloui-Bejaoui, N., El Gharsalli, R., White, D. M., Stokes, N. A., & Burreson, E. M. (2010). Observation of a Bonamia sp. infecting the oyster Ostrea stentina in Tunisia, and a consideration of its phylogenetic affinities. *Journal of Invertebrate Pathology*, *103*(3), 179-185.

Hill, K. M., Stokes, N. A., Webb, S. C., Hine, P. M., Kroeck, M. A., Moore, J. D., ... & Carnegie, R. B. (2014). Phylogenetics of Bonamia parasites based on small subunit and internal transcribed spacer region ribosomal DNA sequence data. *Diseases of aquatic organisms*, *110*(1-2), 33-54.

WOAH (2024). Manual of Diagnostic Tests for Aquatic Animals, section 2.4. Diseases of molluscs, Chapter 2.4.0 General information and disease-specific chapters*)*: https://www.woah.org/en/what-we-do/standards/codes-and-manuals/aquatic-manual-online-access/

3. Equipment, reagents and environmental conditions

Refer to SOP "Detection of mollusc pathogens by *In Situ* Hybridization (ISH)" and to SOP "B*onamia* spp detection by Polymerase Chain Reaction and species characterisation by Restriction Fragment Length Polymorphism (PCR-RFLP)" for PCR conditions used to produce the *Bonamia* sp. probe.

4. Procedure

4.1. ISH probes

ISH probes are labelled with digoxigenin (DIG)

Targeted pathogens and gene region	Reference	Probe type	Probe name and sequence
Bonamia spp. and several members of *Haplosporidia* (18S)	Cochennec et al. 2000	Labelled PCR amplicon	BO-BOAS PCR product (300bp)
B. ostreae (18S)	Hill et al. 2014	Labelled oligonucleotide	Bost171: CCGCCGAGGCAGGGTTTGT

B. exitiosa and closely related *Bonamia* sp. (18S)	Hill et al. 2010	Labelled oligonucleotides (cocktail of 3)	CaBon166: CGAGCAGGGTTTGTCACGTAT CaBon461: TTCCGAATAGGCAACCGA AG CaBon1704 :CAAAGCTTCTAAGAACGCGCC

Note: specificity of species-specific probes was evaluated on a limited number of samples (EURL technical report 2021-2022).

4.1.1. Preparation of PCR-based probe

The probe is prepared by PCR with DNA from bivalve highly infected with *Bonamia* sp.. DNA is extracted from a piece of tissue (generally a pool of gills, mantle and digestive gland).
The probe is prepared as described in the SOP "*Bonamia* spp detection by Polymerase Chain Reaction and species characterisation by Restriction Fragment Length Polymorphism". The only difference is the addition of Dig labelled dUTP (Roche) in the PCR Mix.

Primers for the preparation of the *Bonamia* sp. probe:

Bo: 5'-CAT-TTA-ATT-GGT-CGG-GCC-GC-3'
BoAS: 5'-CTG-ATC-GTC-TTC-GATCCC-CC-3'

PCR are prepared for a total volume of 100µl of probe. **PCR Mix** for each tube is:

	Volume per tube	**Final concentration**
Buffer (10X) *	10 µl	1X
$MgCl_2$ (25 mM) *	10 µl	2.5 mM
dNTP (2mM)	10 µl	0,2 mM
Dig Labelled dUTP (1 mM)	2.5 µl	0.025 mM
Bo (100 µM)	1 µl	1 µM
BoAS (100 µM)	1 µl	1 µM
Taq polymérase (5U/µl)	1 µl	5 U
dH_2O	64.5 µl	

- 98 µl of this PCR mix is dispensed in each PCR tube
- 2 µl of extracted DNA (100 ng/µl) is added to each tube

**Those parameters may vary depending on the commercial PCR mix used*

Control of the labelling efficiency
DNA sample should be amplified in parallel without the addition of DIG-labelled dUTP. PCR products with and without DIG-labelled dUTP are compared on a gel electrophoresis to control for the DIG labelling. The labelled PCR product should migrate slower and appear larger than the unlabelled PCR product.

4.1.2. Preparation of oligonucleotide-based probe

Pathogen-specific oligonucleotide probes are to be purchased with a 5'end digoxigenin label (for example from Eurogentec).

4.2. Hybridization

Refer to SOP "Detection of mollusc pathogens by *In Situ* Hybridization (ISH)

Two types of control are used:

- **Negative** controls consist of sample tissue without probe (for each sample to analyse, prepare one slide with probe and one slides without probe).
- **Positive** controls consist of tissue sections from controlled **infected** oysters or mussels
- **The *Bonamia* sp. probe** can be used as positive control to check the integrity of DNA on analysed slides.

4.3. Interpretation

Validation of the ISH assay

- Negative controls must appear negative.
- Positive controls must appear positive.
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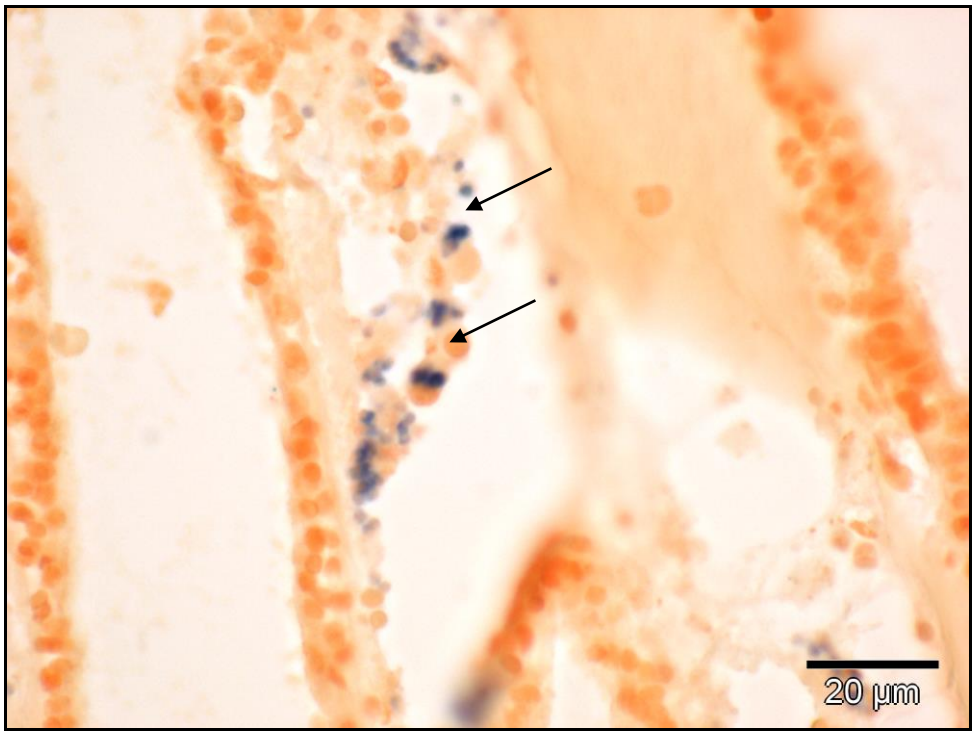
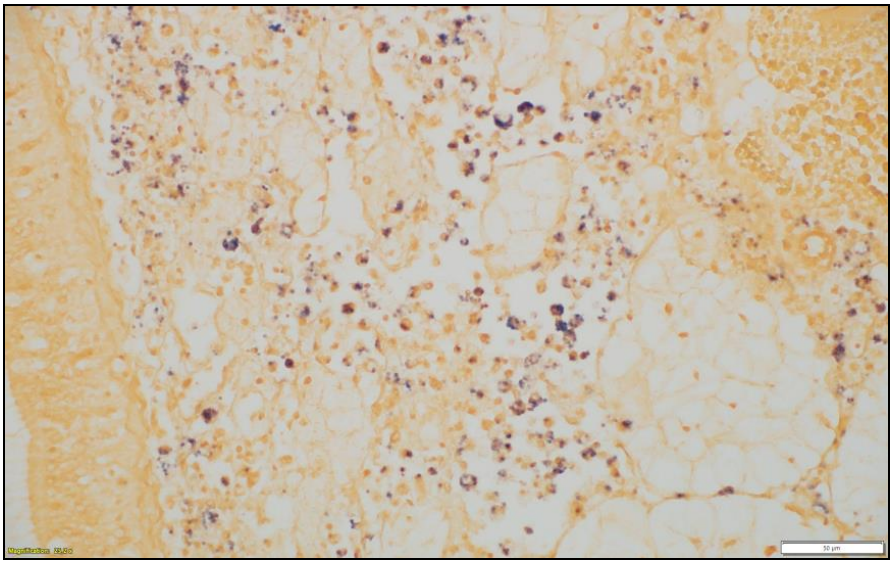
Interpretation of sample results

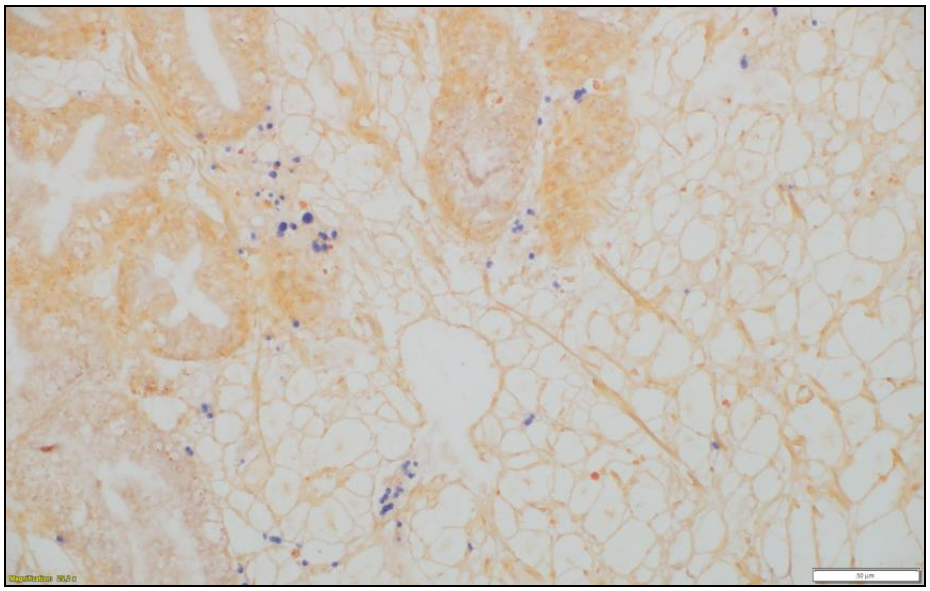
- Slides are checked for positive signal looking like dark purple spots in hemocytes (see picture below).
- A positive signal in an individual detected positive by histology allows concluding that it is an infection with *Bonamia* sp.
- A positive signal in an individual detected positive by histology with the Bost171 probe is indicative of an infection with *B. ostreae*
- A positive signal in an individual detected positive by histology with the Cabon probes is indicative of an infection with *B. exitiosa*



Picture: Flat oyster *Ostrea edulis* infected with *Bonamia* sp. tested by *in situ* hybridization. The parasites appear as dark purple spots in the connective tissue and in haemocytes of the gill (arrows).



Picture: Flat oyster *Ostrea edulis* infected with *B.ostreae* tested by *in situ* hybridization with the Bost171 probe.



Picture: Flat oyster *O. chilensis* infected with *B.exitiosa* tested by *in situ* hybridization with the Cabon probes